

# The last regional particle accelerator?

**Next week's meeting of the CERN Council will not, after all, provide the full-throated endorsement of the Large Hadron Collider for which physicists had been hoping. But cautious governments should restrain misanthropy.**

EUROPE'S high-energy physics community will know in the next few weeks whether it will be able to build the Large Hadron Collider (LHC) on which it has set its heart. The meeting of the CERN Council next week will be decisive. But expectations that governments party to the CERN treaty, which have been saying that they are "in principle" in favour of the project, would instruct their delegations to make a polite expression of enthusiasm are likely to be disappointed. Britain and Germany (see page 509), acting in concert, hope also to use the council meeting to change the rules by which the annual budget of CERN is fixed. The fear is that the outcome will be that the annual budget to be fixed for 1995 (roughly SFr970 million) will remain unchanged (in Swiss francs) for the following decade, with no allowance for inflation. That, if seriously intended, will be a bad business.

Nobody suggests, of course, that the world (let alone Europe by itself) owes high-energy physics a living. Or that the cost of installing the LHC (in the tunnel built for the electron-positron collider LEP), at roughly US\$2 billion over ten years, is a trivial sum. (CERN had planned to finance three-quarters of the cost from its regular budget, but had been hoping for an extra SFr500 million in the closing years of this decade.) On the contrary, there is every reason to fear that, whatever governments may say to the contrary, national contributions to CERN will diminish what is otherwise available for less esoteric research projects. But CERN's case for the LHC deserves sympathetic attention, particularly now that the high-energy physics community in the United States has been robbed of the Superconducting Super Collider (SSC).

The case for high-energy physics is what its practitioners say: it is the way to learn how matter is constructed, and from

what. Thirty years of sustained effort have produced what is called the Standard Model, that in which the ingredients of matter are quarks and fermions, in which forces between particles are mediated not just by photons and gluons but by intermediate vector bosons (found at CERN in 1990) and in which the shadowy Higgs boson is, in effect, the yardstick of mass. It is not necessary to share the injudicious view that high-energy physics will be done when the Higgs boson has been demonstrated among the products of a sufficiently energetic collision to regard that demonstration as a fit crown for an international intellectual endeavour that has already changed the way the world seems.

Those who hold the purse-strings could, of course, have decided that \$2 billion is a lot to pay to confirm high-energy physicists in their belief that they have been right about the Standard Model all along. (That sense of contentment will be more persuasive when there is more sense to be made of the quantization of gravity, among other things.) But that appears not to be the case. CERN's members, rather, seem ready to let LHC come into being, but some of them appear to insist that it should be built within a budget that declines in real terms. The danger, of course, is that the result will be a botched job. CERN has an excellent reputation for building accelerators to specification, on time and within budget. Skimped engineering could put an end to that. And the old trick of stretching the construction schedule is a recipe for diluting the enthusiasm of the engineers on whom the performance of the LHC will hang.

But why should not the United States make good any shortfall there may be? That beguiling notion is based on a thorough misunderstanding of last year's cancellation by the US Congress of the SSC project, more than four times as expensive as the LHC. There is nothing to suggest that the Congress had not grasped the arguments about the Standard Model. Instead, it took the view that the proposed test of it was unaffordable. Period. Is it likely that the same Congress, with all the enemies it has made in Texas, will change its mind in favour of a project in Geneva?

That is why the best outcome of next week's meeting will be a fudge. Britain and Germany are within their rights to ask for a budget ceiling, but wrong to insist that it must be settled here and now. Better to wait for a couple of years to tell which way the wind is blowing then. On relations with the United States, CERN as a laboratory should be encouraged to keep alive the transatlantic collaboration that has brought high-energy physics to its present state. Euro-chauvinism is the

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last thing the field needs. Meanwhile, there are even more distant issues needing urgent attention. What (if any) accelerator will succeed LHC, and how (and where) would it be built? Now is the time to forge a thorough international enterprise to consider those thorny questions. And what, in 2010 or thereabouts, will happen to the CERN laboratory itself? If Britain and Germany are hoping for an answer to that question by their demand for budget ceilings, they would be better advised to take it up directly. On the time-scale of accelerator construction, 2010 is indistinguishable from the year after next. □

## US health reform falters

**The US Congress is in bitter dispute about health reform and the proposed employer mandates.**

THE debate about health-care reform in the United States is at once moving apace and in danger of stumbling to a stalemate as various pieces of legislation emerge from the many congressional committees with jurisdiction of one sort or another. Two main issues divide the Congress, sufficiently to suggest that US President Bill Clinton's plan has little chance of becoming law. Clinton has predicated affordable universal coverage on two ideas: first, that employers should pay 80 per cent of employees' health insurance and, second, that the entire country should be divided into giant regional alliances from which people would purchase insurance, which is an untested notion. Republicans, with the support of some Democrats, are virulent in their opposition to the so-called employer-mandate, which they believe would put small business out of business. And the Rube Goldberg plan for regional alliances has few, if any, real supporters outside the White House.

The indictment for fraud of Representative Dan Rostenkowski (Democrat, Illinois) has been a further setback. When he was ousted last week from the chairmanship of the House of Representatives' Ways and Means Committee, whose vote is crucial to the passage of any bill, Hillary Rodham Clinton, the president's wife, worried aloud that the loss of his political clout will mean that a means of forcing congressmen to support the president's plan has vanished. But the present jumble of bills, voices and warring camps suggests that it will take more than one man to get health-care reform legislation through this Congress before it adjourns for elections in the fall.

Within the past couple of days, several influential participants in the debate have, for the first time, admitted that there may be no bill at all. Representative John Dingell (Democrat, Michigan), who is known for his power to bring his Oversight and Investigations Committee into line, cannot muster the votes for a bill with an employer mandate and has said he would prefer no bill to a bad one. On the other side, Republican leader Robert Dole said last week that he is prepared to turn the fall elections into a *de facto* referendum on the form health-care reform should take.

For those whose chief concern is how health-care reform will affect (and even damage) academic research institutions, delay may well be the best outcome. A provision in some bills to require 50 per cent of US physicians to be trained in general practice by early in the next century is ill thought out and should be dropped.

Other provisions to tax health insurance premiums or to single out other federal resources to support teaching and research hospitals have survived in some bills, but have been knocked out of others, in the usual hurly-burly of political horse-trading. But this is an issue of vital importance to the US research enterprise, even if it is not at the top of the public agenda. (Research seldom is.) A bill whose funding provisions would effectively put research institutions out of business is not in anybody's interest. As things are, no bill at all would be best for now. □

## ... and in Britain also

**Britain's internal market in health care has made problems for research.**

HISTORIANS will marvel, but will not be surprised, that the difficulties arising in the United States on the financing of medical research are almost exactly mirrored in Britain. After all, there is a close analogy between the 'regional alliances' Clinton advocates as purchasers of health care on behalf of insured people and the regional health authorities that now do that in Britain (with public funds) on behalf of patients of the National Health Service (NHS).

But the British government has manoeuvred itself into an ideological contradiction. The reorganization of the NHS in the past three years has been driven by the belief that the interaction between purchasers of health care and its providers (hospitals, for example) would create an efficient internal market. (The arrangement is to some degree complicated by the encouragement of physicians to function both as providers and purchasers.) Logic then requires that the purchasers should also purchase research. Sadly, the thought seems not to have crossed the minds of the health authorities, nor (it appears) of the government.

The institutions chiefly affected by this neglect are Britain's teaching hospitals, which are financed from two sources — from the NHS (in respect of patient care) and from the Universities' Funding Councils (in respect of the education they provide to physicians). But now the teaching hospitals must compete with others for patients on the internal market, while the traditional use of university funds to support research has been undermined by the doctrine (spreading through the rest of British academic research) that researchers should be accountable to their sponsors project by project. So, inevitably, there has been a committee (see page 514). Not unusually, the government appears to be embarrassed by its conclusions, and is sitting on them. But the issue of British health research is too important to be the victim of the government's wish that a problem it has made for itself would melt away. □



# UK and Germany put the squeeze on CERN's plans for new accelerator

**Munich.** Last-minute attempts by the British and German governments to impose stringent financial conditions on the construction of Europe's planned Large Hadron Collider (LHC) may bring an element of brinkmanship to next Friday's (24 June) meeting of the council of the European Laboratory for Particle Physics (CERN) in Geneva.

With all 19 member states in favour — at least in principle — of proceeding with the LHC it is unlikely that the council will block approval for the project. The demise of the US Superconducting Super Collider (SSC) means that the LHC would be the world's largest particle accelerator and its best prospect for detecting predicted fundamental particles such as the Higgs boson.

But such approval will not be as automatic as some had hoped, because the CERN management has so far dismissed as unacceptable a German/British proposal to change a vital voting rule for agreeing on the inflation-linked rises in annual subscriptions from member states.

At present such rises, needed to cover the additional costs of personnel and materials,

have to be agreed by a simple majority, although under an amendment introduced two years ago the countries in favour of an increase must contribute more than 55 per cent of the total budget. This rule is intended to ensure that the growing number of smaller member states cannot block the wishes of CERN's main contributors, namely Germany (which provides 22.5 per cent of the budget), France (17.5 per cent), Italy (15.5 per cent), the United Kingdom (13.5 per cent) and Spain (7.5 per cent).

Citing financial pressures at home, both Britain and Germany now want an individual right of veto, and are pressing for a rule requiring any inflation-linked rise to be agreed unanimously. But such a move is unlikely to be welcomed by many other member states, particularly because it would also give a veto to small member countries.

Furthermore, given the expected difficulty in reaching unanimous agreement, the move could effectively hold the budget level in cash terms at 1995 prices for the whole of the LHC construction programme, and the first two years of experiments (1995–2005). This could reduce the purchasing power of



the budget by 30 per cent at a time when CERN already has a large shortfall in cash.

Under CERN's proposed construction programme, the LHC will cost an estimated 2.6 billion Swiss francs (US\$1.8 billion), SFr500 million more than the member states would pay out of their normal, inflation-linked contributions. Christopher Llewellyn Smith, the laboratory's director-general, proposes to make up this shortfall in one of two ways (see *Nature* 366, 714; 1993).

The preferred option would be to raise the money from non-member countries, particularly the United States, Japan and Canada, whose own particle physics programmes have been left in the lurch by the demise of the SSC. Positive signals have come from these countries, particularly from physicists in the United States (see *Nature* 369, 266; 1994); but none want to start negotiations until the LHC has been formally approved.

Lacking such contributions, the second option would be to extend the construction phase by two years, delaying the start of experiments until the beginning of 2005. But CERN is keen to avoid such a delay, as experiments on the current CERN accelerator (LEP) will come to an end in 1999.

Llewellyn Smith wants next week's council meeting to take a clear decision on initiating the construction programme so that he can put these uncertainties to rest. The decision does not have to be unanimous. But CERN is keen for a public show of harmony, as the non-member states it is courting will be present at the meeting as observers.

The final days before the meeting are therefore likely to see some hard bargaining between Llewellyn Smith and the British and German delegations. One compromise could be a temporary agreement to fix contributions in cash terms, in exchange for further trimming of CERN costs — ►

## ... as Spain gets cut-rate membership

**Munich.** A long-standing dispute between Spain and CERN over Spain's membership subscription is likely to be resolved next week with an agreement to give the country a discount averaging 23 per cent over the next five years.

Spain has withheld payments for over two years. It argues that, because of a weak domestic base in particle physics, it has too few staff and visiting scientists in Geneva, and wins less than one per cent of the industrial contracts issued each year, despite contributing 7.5 per cent of the laboratory's budget.

Sympathetic to Spain's severe economic problems, CERN had offered a 20 per cent discount in its subscription over the next five years — provided that it paid its existing debts. Spain is now offering a compromise believed to be acceptable to CERN, under which it will pay its debt by underwriting a bank loan to CERN.

In exchange, Spain will be allowed a decreasing reduction on its subscription over the next five years, starting at 40 per cent in 1994 and ending with 10 per cent in 1998. Spain is to consider setting up a research institute for particle physics.

Six other countries also have a special agreement for reduced subscriptions, loosely based on gross domestic product.

The four new members from central Europe — Poland, Hungary and the Czech and Slovak republics — pay only token contributions. Negotiations are taking place to bring them up to full contribution by the year 2000. But even then they are likely to contribute only a few per cent of the total budget of the LHC.

Greece negotiated a 60 per cent reduction in its subscription in the 1970s by arguing that its scientific community was so weak that it reaped little benefit from its membership. But now both sides agree that Greece's particle physics community has prospered, thanks to training at CERN, and Greece has agreed to increase its contribution, aiming to reach its full level by 2001.

But the most important case is Germany, whose contribution should have risen after reunification. But because the costs of reunification were themselves so high, CERN agreed that Germany's contribution should remain unchanged (at 22.5 per cent of the budget) until 1995, with a possible extension of a further two years. The extension is likely to be a very sensitive issue when it comes up for discussion this year with countries such as France likely to object strongly to any further concessions.

**Alison Abbott**



perhaps by limiting experiments involving the laboratory's smaller accelerators — until the project is reviewed in 1997, when final decisions on the timetable need to be made.

At present, however, neither Britain nor Germany seems willing to drop their demands for new voting procedures. And other countries are worried that this hard-line position could jeopardize the final agreement.

French officials, for example, say they will be looking at the two countries' proposals "with concern" as they want the LHC decision to be made with full assurances of financing. Italy is also worried. "We would regret seeing the buying power of CERN decrease," says Claudio Orsalesi, adviser to the Italian delegation.

Meanwhile, CERN's two host countries, Switzerland and France (the laboratory straddles the border between the two) have announced they are each prepared to accept another UK/German-led demand put forward last year, namely that they should make an increased payment towards the LHC in recognition of the benefits that CERN brings to their local communities.

Both countries say they will make a one-off contribution after 1997 — either in cash or in kind. Switzerland will probably put forward a concrete proposal next week; the French government is still negotiating with the local authority where CERN is based to agree a sharing of cost. **Allison Abbott**

## IBM physicist picked for Trieste centre

London. **Praveen Chaudhari** (below), a senior physicist at IBM's Thomas Watson Research Centre in Yorktown Heights, and a former head of the company's science programmes, has been chosen as the new director of the International Centre for Theoretical Physics in Trieste.

**Chaudhari** has been with IBM since 1966. He succeeds **Abdus Salam**, who has been director of the centre — aimed in particular at providing training courses for physicists from developing countries — since 1964, and retired at the beginning of this year.

**Chaudhari** is the author of more than 150 technical papers, and is widely known for his work on electronic materials. He has been a senior advisor to both the US and Indian governments, and was chosen from a list of about 20 candidates by the governing council of the ICTP at a meeting held last week at the International Atomic Energy Agency in Vienna. □



# US physicists urged to build links with the modern world

**San Francisco.** A leading US physicist has warned his colleagues that the physical science community is seen as "non-cooperative with a new move to connect science more closely to the needs of society". In response, professional societies will be urged to endorse mission statements emphasizing their contribution to the "long-term opportunity for the nation".

**Burt Richter**, director of the Stanford Linear Research Center (SLAC) and president of the American Physical Society, told a forum of senior physical scientists earlier this month: "We are seen as recalcitrant, and wanting to go our own way."

In an attempt to correct this impression, the National Research Council (NRC) — the research arm of the National Academy of Sciences — has embarked on an attempt to forge a constructive response from the mathematical and physical sciences to their changing social environment. To start the process rolling, the NRC has sponsored a series of round-table meetings in Virginia, Colorado and California, aimed at hammering out a fresh justification for the American public's investment in the physical sciences to replace the old, unstated rationale of winning the Cold War.

At the Californian meeting, co-sponsored by Stanford University and held in San Francisco, senior academics in mathematics, physics and chemistry were invited to join the heads of government laboratories, Silicon Valley entrepreneurs and White House and congressional staff. Five important themes emerged from a weekend of discussion:

- The physical sciences community is essentially inward-looking. Even its senior members are happier dealing with internal issues, such as course structure, rather than external ones, such as why the federal government should continue to pay its bills;
- The community does face very real external threats, in particular the desire of its paymasters to 'pick winners' and to apply quantitative measures to the returns on investment in research investment;
- The community is sharply divided in its response. Some believe that the US research enterprise is so finely tuned that tampering with it can only do harm; others welcome the external pressure brought by the end of the Cold War as an overdue opportunity for radical change;
- Change, whether radical or gradual, will require physicists, chemists and mathematicians to work with other disciplines, including computer science, engineering and, in particular, the life sciences;
- Change will eventually take university teachers in mathematics, physics and chem-

istry down the road already trodden by the engineering schools, towards more practical course content and industrial experience for postgraduate students.

But even senior faculty from elite schools represented at the meeting were at times uncomfortable discussing the big issues of science policy. A session on the "social contract" between science and society, for example, ended after two minutes when one speaker claimed that no such contract existed.

**Bill Harris**, head of mathematics and physical sciences at the National Science Foundation, identified one consequence of the community's isolationist tendency. "We sit on the sidelines of this process called democracy," he says. Such non-participation, others pointed out, can extend to the denigration of scientists who follow career paths outside academic institutions.

But outside pressures coming to bear on the physical sciences are not always obvious. MRC Greenwood of the White House science policy office warned, for example, that government performance law coming into force in 1997 will require federal agencies to formally assess programme outputs. "If the science community doesn't participate in developing appropriate assessments, it will become subject to measurements developed by or appropriate to other government activities," she says.

**Richard Zare**, the Stanford chemist who organized the meeting, favours a balanced response to all these pressures: "If we don't respond, we're dead; if we respond too much, we wreck the enterprise," he says. A rapid response is favoured by those in newer disciplines, such as computer science, who disliked the predominance of mathematics and physics in the old, Cold War system.

The Silicon Valley people at the meeting were generally happy with their current relationships with university science — and with the steady output of a postgraduate education system that they consider unequalled anywhere in the world.

But if the physical sciences have no trouble relating to industry, their relationship with a wider society is more fraught. In one attempt to reach out, **Richter** and **Robert Byer**, an applied physicist at Stanford who is president of the Optical Society of America, pledged to get the boards of their respective societies to consider mission statements.

One proposed wording is that "[the society] will focus its capabilities on teaching, research and scholarship to generate knowledge and long term opportunity for the nation". It sounds a small step, but it would be a big leap for scientists who tend to see their role as confined to the pursuit of pure knowledge. **Colin MacIlwain**

# Budget freeze taints French science plans

**Paris.** With only a few days to go before France's science minister, François Fillon, presents his plans for French research to the National Assembly, the finance ministry has pulled the rug from under his feet by freezing part of the budget for civil research and development.

The freeze may amount to as much as FF400 million (US\$68 million) in cash payments and FF500 in programme authorizations, equivalent to 8 per cent of the total

research and development budget (excluding salaries).

The total promised research budget in 1994, at FF51.6 billion, was itself only 1.2 per cent up on the revised budget for 1993, and 2 per cent down on the original. Last year's revision cut cash payments by FF288 million, and programme authorizations by FF795 million.

Research organizations say it is too soon to predict the impact of the freeze, as they

have not yet been told how it will be distributed among individual agencies. But the Centre National de la Recherche Scientifique (CNRS), for example, spends more than three-quarters of its budget on salaries, and the freeze will therefore shrink still further the amount available for launching new research programmes and initiatives.

Researchers are also annoyed that the freeze comes half-way through the year, and therefore requires acrobatic accounting to limit the damage. Fillon says that a freeze is not a cancellation, and that he wants to restore the frozen funding. But observers in Paris point out that freezes rarely "thaw", and usually amount to straightforward cuts.

The move comes at a particularly bad moment for Fillon, a week before he presents his plans for research to the National Assembly. Fillon has argued that the spending cuts are unrelated to his plans for research. But the question of funding now threatens to overshadow next week's debate.

Moreover, the finance ministry's action may also damage the credibility of the science minister, who recently assured the research community, following a six-month national 'consultation' on research (see *Nature* 368, 675; 1994), that spending on science would be maintained. Some are predicting that Fillon may seek a reduction in the freeze before next week's debate, in an effort to show himself as a defender of the scientific community.

In the longer term, the freeze raises questions about the government's commitment to science. Some scientists see the spectre of 1986, when research bore the brunt of more than half the cuts in spending by the incoming neo-Gaullist government, led by Jacques Chirac. It annulled the research budget of the previous socialist government, and held spending by the research organizations virtually constant.

Indeed, the finance ministry may see research as an easy target for cuts. The current freeze stems from the government's need to make public spending cuts of FF7 billion. In particular, the government is thought to be planning a reduction in interest rates in order to revive the economy, and therefore needs both to reduce the deficit and to prop up the franc to reassure the financial markets.

The freeze is not necessarily a bad omen for the research budget for 1995. The government is taking unpopular measures before next year's presidential elections. Edouard Balladur, the prime minister and a possible presidential candidate, may increase public spending overall in next year's budget to build public confidence in his administration. But some cynics point out that this would not preclude the government from suddenly 'revising' the budget after the election.

**Declan Butler**

## Soap giants trade blows over tests

**London.** Two of the world's largest soap powder manufacturers have become locked in a war of words and figures over allegations of fabric damage caused by low-temperature detergents containing a new manganese-based catalyst.

Last month, the Anglo-Dutch company Unilever launched a range of products containing the catalyst, which removes stains faster and at lower temperatures than older detergents. The catalyst is covered by more than 30 patents, and was developed at a reputed cost of more than £100 million.

Marketed in the United Kingdom as Persil Power and as Omo Power in the rest of Europe, the new catalyst is a heterocyclic complex of manganese that promotes the selective oxidation of stains by hydrogen peroxide.

Unilever claims that the catalyst is good news for the environment, as not only do low temperature washes save energy, but the production of the detergent requires both less energy and less water than traditional manufacturing methods.

But the company's US-based rival Procter & Gamble, which manufactures Ariel, a leading soap powder, quickly claimed that the catalyst caused damage to garments under certain washing conditions.

The Dutch company was equally quick to take the US company to court, claiming it had made "untruthful and misleading statements". It subsequently dropped the charges, admitting that Persil Power could cause damage under "extreme conditions".

But the truce did not last long. Last week, Procter & Gamble backed up its initial charges with a series of research reports from six independent European institutes apparently endorsing its conclusions, and producing pictures of faded and shredded garments allegedly washed in Persil Power.

Procter & Gamble quoted excerpts from the reports saying that the fabric damage

caused by the new formulations to cotton fabrics is "beyond the tolerances used to date for household detergents" and that this is "accelerated by certain dyes which can lead to holes well within the expected lifetime of the garment".

Unilever acknowledges that it has re-

IMAGE  
UNAVAILABLE  
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REASONS

**Washday blues:** Procter & Gamble's 'evidence' (left) of the hazards of rival Unilever's new detergent.

duced the amount of manganese catalyst in its products. But the company says a little "fine-tuning" is normal in a product launch.

At the same time, the Anglo-Dutch company has challenged the results being quoted by its rival from one of the six institutes, the British Textile and Technology Group. In tests commissioned by Procter & Gamble, the institute reported that Persil Power "totally destroyed" some garments after 50 wash cycles. But in separate tests commissioned by Unilever, the same institute found "no physical damage visible" on a range of articles after 15 and 25 washes.

"If you pick the right combination of dye, stains, material and basic colour, you can get results which will show severe damage," says Andrew Seth, chief executive of Unilever's UK detergents business. "If I wanted to, I could do the same with competing products."

The company has pledged to reimburse any customers who can prove their clothes have been damaged by Persil Power. Britain's Consumer Association is to carry out its own independent tests of the powders, and plans to produce a report in July.

**Maggie Verrall**



## Italian researchers in bid for fairer promotion rules

**Munich.** A 3,000-strong body representing Italian scientists has asked the government to introduce changes to the system of academic appointment, which it claims is open to corruption and abuse.

The Italian Federation of University Researchers has asked the cultural committees of Italy's new upper and lower houses, as well as new minister for universities and research, Stefano Podestà, for meetings to discuss the introduction of more rigorous assessment of applicants' academic qualifications.

Tenured academic positions in Italy are not awarded by individual universities, but through open *concorsi* (competitions) organized every few years by the ministry of research and universities in Rome. Applicants go through two stages. First, their academic achievements are considered by a subject committee. Short-listed candidates are then required to attend an oral examination, after which successful candidates are allocated to universities by the ministry.

Critics of the system complain that it is open to abuse, as there are no fixed criteria for the assessment made at either stage. They also say that members of assessment committees often use their personal influence to arrange support for particular candidates (see *Nature* 366, 293; 1994).

One result is that Italians who have worked abroad often find it difficult to get back into the system. This undermines the effectiveness of a 1985 modification to the *concorsi* law, which gave universities the right to apply for a 5 per cent increase in posts to attract academics working abroad back to Italy.

Carlo Bulletti, secretary of the federation, says it wants to see agreement on a set of minimum criteria for passing the first written stage of a *concorso*. According to Bulletti, too many people with poor publication records pass this stage, and too many of those are subsequently given posts.

Last December, says Bulletti, the cultural committee of the senate expressed its support for the proposed changes, but the government was near the end of its term and little could be done. He is optimistic that the new administration, elected on an anti-corruption ticket, will be keen to tighten up the *concorsi* system by reducing the impact of personal influence on appointments.

The federation is planning an international workshop in December to define the criteria used to assess publication records — including both the number of publications and their impact — that it would like to see introduced into the first stage of the *concorso*.

The government's unfulfilled promise to

help native scientists working abroad to find full-time posts in Italian universities has worked out particularly badly for Canio Vosa, a botanist who was employed for 20 years in the plant sciences department of the University of Oxford in the United Kingdom.

In 1989, Vosa was offered a chair of botany at the University of Pisa under the government 'expatriate' rule. Vosa was in fact entitled to a professorship, having successfully participated in a *concorso* in 1979, and being subsequently offered a job at the University of Rome.

Vosa left his post at Oxford in March 1991 and moved into a room and laboratory assigned to him at the University of Pisa. A few months later he was informed by the ministry of universities and research that his post at the top of the lecturer scale at Oxford did not fulfil the criteria of the new rule, which was restricted to senior lecturers only.

Despite written clarification of his former position by the University of Oxford — which has only a single scale for lecturers and senior lecturers — and petitioning by the University of Pisa, Vosa has lost his chair in Pisa and is now out of work.

**Alison Abbott**

## US science to pay price if station proceeds

**Washington.** Congress is ready to make science pay the price if the Clinton administration succeeds in bulldozing the increasingly friendless international space station through this summer's budget negotiations.

Actions by congressional committees over the past week suggest that the National Science Foundation (NSF) can expect at best an increase lower than the expected rate of inflation in the next financial year. The National Aeronautics and Space Administration (NASA) may be forced to make deep cuts in its science programme to keep the space station alive.

The NSF receives its money from the same Congressional pot, overseen by the veterans affairs, housing and urban development and independent agencies appropriations subcommittee, as NASA — and thus the space station.

The bad news for the science agency has come from the House subcommittee, chaired by Louis Stokes (Democrat, Ohio), which passed a budget giving it an overall increase of 3 per cent, compared to the 6 per cent requested by the administration. As Congress wants more NSF money spent on new buildings and equipment, the agency's research budget would grow by just \$53 million, against \$185 million requested.

## Canada cuts role in space station, but boosts overall plans

**Quebec.** Canada has announced details of a new long-term space plan that will allow it to reduce its financial contribution to the US international space station while maintaining full partnership along with the other 10 countries involved. But its rights to use the space station have, in return, been slightly reduced.

According to John Manley, the minister for industry, negotiations with the US National Aeronautics and Space Administration have resulted in a reduction of Canadian funding from a total of C\$1.2 billion (US\$880 million) to C\$0.5 billion over the next ten years.

Canada's overall space budget, on the other hand, has been increased to a C\$2.7 billion over the same period. On top of C\$1.7 billion of previously approved funds and a C\$800 million increase in last February's budget, this includes a further C\$200 million of new funds.

"As a result of this plan, Canada will by the year 2005 have four new satellites in orbit, Canadian astronauts will have participated in at least five shuttle missions, and Canadian robotics will have played a crucial role in the assembly of the space station," said Manley.

**David Spurgeon**

These figures are bad news for NSF grant applicants, who already face a squeeze as their numbers expand against fixed budget ceilings. But the equivalent senate subcommittee, chaired by Barbara Mikulski (Democrat, Maryland) could produce even lower figures when it votes next month.

Last week, the senate subcommittee threatened NASA with a budget of \$13.7 billion, a drastic \$600 million short of the space agency's request. It also asked Dan Goldin, the NASA administrator, to choose whether to make such a cut by closing the Cassini mission to Saturn or the Advanced X-ray Astrophysics Facility (AXAF). Goldin will report back next week.

But some see Mikulski's \$13.7 billion as less of a serious proposal than a cry for help. If NASA's budget is set so low, House science committee chairman George Brown (Democrat, California) has said he will withdraw backing from the space station to save the rest of NASA's science programmes.

Mikulski, Brown and vice-president Al Gore — all space station supporters — have two weeks to sort out their differences before Tim Roemer (Democrat, Indiana) tries to kill the station on the House floor. Last year, it survived by 216 votes to 215.

**Colin Macilwain**



# 'Misconduct' definitions still prove elusive

**Washington.** The most notable aspect of a two-day meeting on scientific conduct last week in Washington, DC, was a striking sense of *déjà vu*. After more than a decade of intense debate, government rule-making, and at least a dozen reports from professional groups throughout the United States, the meeting remained dominated by the lament that scientific misconduct is hard to define, and even harder to deal with.

The meeting was arranged by the US National Academy of Sciences, the National Academy of Engineering and the Institute of Medicine. One major issue raised was the need for a uniform — and uniformly agreed — definition of misconduct.

The US Public Health Service (PHS), whose rules govern the National Institutes of Health (NIH), operates under a strict definition that includes fabrication, falsification and plagiarism, often referred to as 'FFP'. The PHS also follows a controversial procedure for responding to allegations of misconduct in which the officials conducting the investigation also adjudicate.

By contrast, the National Science Foundation (NSF) separates the investigation from adjudication, but uses an expanded definition of misconduct that includes not only FFP but also "other practices that seriously deviate from those commonly accepted in

the scientific community".

The argument against the NSF definition was put persuasively by Howard Schachman of the University of California at Berkeley, recently appointed as integrity ombudsman to the director of NIH.

Schachman said that the federal government, with its power to ban scientists from the research establishment by denying them jobs or grants, should become involved only in potentially criminal offences. The fabrication of data in a grant application, for example, would constitute an attempt to defraud the government.

Others claimed that the research community should be concerned with so-called deviations from accepted practice, and thus favour the NSF's definition. But if an example cited by Neal Lane, the NSF director, offers any insight — namely the secret switching of reagents in a colleague's laboratory — in practice the agency follows a strict definition of "serious deviation".

The core issue faced by the meeting was how to ensure, within the highly competitive environment of modern science, adherence to the standards of ethical behaviour and scientific etiquette that are said to have characterized a previous era.

Authorship practices were one example of the dilemma that received close attention.

Nearly everyone present agreed that honorary authorship does not promote high ethical standards. But no one could agree just what the proper requirements for authorship should be.

The meeting was therefore left with unanswered questions. For example, if a laboratory director provides space, support and equipment to a student who is not working on one of the director's own projects, should the director still appear as an author on subsequent publications?

Despite efforts by various groups to establish guidelines for recording and maintaining data, this issue also provoked discussion. How should notebooks be kept? Who owns the data? And who should have access to them?

There are usually official answers to such questions. Universities, for instance, tend to be the legal owners of data, as it is the university, not the scientist, that usually receives a grant. But the consensus among the participants was that the area remains a source of confusion.

Not surprisingly, the responsibilities of laboratory heads as mentors also provoked lively discussion. A theme running through the meeting was that the scientific community should hold itself to higher standards of conduct than those written into law. But ways of elaborating and monitoring those higher standards escaped resolution.

That in itself proved to be an interesting (and worrying) observation in the light of the NIH's requirement for universities to give ethics courses to grant recipients, and that the teaching of scientific ethics and etiquette generally has become quite common over the past five years.

Modification of the pressure to publish or perish was put forward by some as an obvious solution. But others felt that, until university tenure committees abandon the practice of evaluating candidates on the basis of quantity rather than quality, it will remain a good but useless adjuration.

Kenneth Ryan of Harvard Medical School (and also chair of the new ethics advisory committee within the Department of Health and Human Services) asked whether either the academy or the Institute of Medicine had mechanisms for expelling members who violate ethical practices.

The answer was that neither does. Indeed, exemplary behaviour is not even a criterion for election to membership of the academy, or indeed of most honorary professional societies.

Bruce Alberts, president of the academy, is faced with digesting the many views expressed at the meeting, and deciding how the academy should lead the way out of the morass of 'scientific integrity' or 'good scientific conduct'. **Barbara J. Culliton**

## ESO seeks new promises from Chile

**Munich.** The European Southern Observatory (ESO) has a summer of hard bargaining ahead if its DM500 million (US\$330 million) Very Large Telescope (VLT) is to be installed, as planned, on Mount Paranal in northern Chile.

The site for the telescope has already been levelled (right). And last month an ESO delegation was reassured by the Chilean foreign minister, Carlos Figueroa, that the Chilean government stands by its contractual agreements, dating back to the early 1960s, guaranteeing ESO privileges that include immunity from local law.

This should mean that ESO is able to ignore recent claims to ownership of the VLT land by a local family, which has disrupted building work and at one point threatened the VLT's future at Mount Paranal (see *Nature* **368**, 676; 1994).

But ESO remains concerned. A statement issued by the organization last week, after the delegation had reported back to the ESO council, said that "unless a clarification of this problem is achieved as soon

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

**Paranal mountain: already levelled to receive the VLT.**

as possible, it is unlikely that the current plan for the construction of the VLT observatory at Paranal can be maintained."

In particular, ESO wants further reassurance from the courts, which have not always given the same interpretation as the government of ESO's legal status in Chile, before it finally decides to go ahead with the planned shipment of the first VLT building. An extraordinary meeting of the ESO council early in August will reassess the situation.

ESO is adding pressure for an early resolution of the dispute by refusing to sign a lengthily negotiated "supplementary treaty" which, among other things, offers privileges to Chilean astronomers. **A. A.**

European Southern Observatory



# Tensions grow over health research proposals

**London.** A fierce struggle is developing between Britain's teaching hospitals, its medical research establishment and the Conservative government over the control of funds allocated for the support of research by the National Health Service (NHS).

At stake is the question of how health-related research should be funded in a political environment where the government is demanding market-style thinking in all NHS activities. Many research-based institutions that have relied on the NHS for support in the past are worried about the consequences for their own survival.

Medical schools are, in particular, concerned about the conclusions of an unpublished report, commissioned last year by the Department of Health and delivered to the government at the end of April. This is expected to suggest that the NHS funds they receive to cover the indirect costs of research should be allocated separately from those provided for teaching, and subjected to far greater scrutiny than at present.

Both teaching and research funds are provided currently through the so-called Special Increments for Teaching and Research (SIFTR), which provides up to 20 per

cent of the funding of teaching hospitals through regional health authorities.

SIFTR money is distributed according to a formula based primarily on clinical student numbers. "The system works, and it works well," says Peter Richards of St Mary's Hospital Medical School in London, chairman of the council of the Deans of UK Medical Schools.

Reforms that made it more difficult to obtain NHS support for research could mean that some university teaching hospitals "could go broke", he warns. Similarly, redirecting research funds to other institutions considered more 'productive' by the government would amount to little more than "robbing Peter to pay Paul".

Both proposals, however, are believed to figure in the report on NHS research prepared by a task force chaired by Anthony Culyer, professor of economics and provice-chancellor of the University of York.

At the same time, the government itself is believed to be unhappy about another conclusion of the report, namely that bodies such as hospital trusts, which it wants to turn into the main decision-makers for allocating NHS funds as the 'purchasers' of health

care, are not well placed to make decisions about what research they should be supporting.

Publication of the detailed proposals made by Culyer on how to separate out the research element of SIFTR funding, and how to organize the 'top-slicing' of money allocated to local bodies for health service research, is still awaiting a decision by the health minister, Brian Mawhinney.

But, speaking at a meeting in London organized by the British Postgraduate Medical Federation, Culyer said he was able to reveal some of the basic principles the drafting group had agreed on, and which underpin its main conclusions.

On the current arrangement for funding teaching hospitals, for example, Culyer said that most of the 200 individuals and institutions who provided evidence to the task force "wanted to see the teaching and research components of SIFTR separated".

Culyer added that it was "widely felt" that the research infrastructure in general practice and health care "generally needed core funding based on university departments and networks of research-oriented community-based practice".

Similarly, there were a number of reasons, he said, why giving health-care purchasers responsibility for commissioning research on top of all their other responsibilities "seemed to us not only completely unrealistic under present circumstances, but probably inappropriate".

He listed four reasons for this conclusion: that the fruits of research and development (R&D) are a 'public good'; that the various skills required to commission research properly are 'very scarce'; that giving a single organization responsibility for both health care and R&D purchasing "would pose a terrible dilemma for local decision makers"; and that local purchasers were rarely on international research networks.

Health-care purchasers needed to be given a significant voice in decisions about the allocation of R&D resources, said Culyer, "but without wrecking the system of rocks and whirlpools I have just described".

Such a conclusion, if it survives in the published version of Culyer's report, is likely to be welcomed by the Medical Research Council. Many MRC scientists have recently been complaining that the reorganization of the health service — and in particular the greater discretionary powers being given to local purchasers — threatens to undermine Britain's traditional strengths in clinical research.

But it is a direct challenge to the thinking of government ministers who have been arguing in favour of raising these powers to a maximum. How far they are prepared to retreat from this position to meet the requirements of research is now being hotly debated in Whitehall.

**David Dickson**

## Biomedical levy gathers pace in US

**Washington.** A one per cent levy on health insurance premiums, which would plough an extra \$5 billion annually into biomedical research, is included in the first health-care reform bill to clear a congressional committee. Officials say it stands a good chance of inclusion in a final bill.

Although the proposal for a levy was widely seen as improbable when launched three months ago (see *Nature* 368, 3; 1994), it has since been gathering momentum in the Senate. It has less support in the House, where key committees — especially that chaired by John Dingell (Democrat, Michigan) — are having great difficulty agreeing any bill at all.

But biomedical research bodies and medical schools backing the levy know that, despite its present chaos over health care, Congress is almost certain to come up with a bill for President Clinton to sign in the autumn, and are increasingly confident that the research levy will be in it.

As Senator Edward Kennedy's labor and human resources committee passed its health-care bill last week, his research levy co-sponsors Tom Harkin (Democrat, Iowa) and Mark Hatfield (Republican, Oregon) called on supporters to mount a final push for the votes of Congressmen on key House committees.

The research levy would take one per cent of all health insurance premiums, and

place it in a fund which would release the money directly to the National Institutes of Health (NIH) — on condition that the institutes' existing appropriations continue to grow (in order to ensure that the new money is genuinely additional). Most of the money would be divided between NIH institutes in proportion to the amount they already receive.

The proposal benefits from simplicity, from a splendid isolation from the rest of the complex, interlocking proposals for health-care reform, and from a strong feeling on Capitol Hill that the health-care package needs to contain some benefits for medical schools. But it suffers from the fact that it smells like a tax.

Hatfield, who describes the measure as "not a new tax *per se*, but a fee in the contract people have with their insurance company", says it has only one real opponent in the Senate, namely finance committee member Dave Durenberger (Republican, Minnesota).

Harkin says he is "very optimistic" that the measure will figure in any final bill. He has resisted calls to scale down the proposal, and claims he has "good support around the country. I don't think any compromises need to be made". Compromises seem certain; but come the autumn, biomedical research may get a pleasant surprise.

**Colin Macilwain**



# Breast cancer prevention trials 'can re-start'

**Washington.** A controversial clinical trial of the effectiveness of the drug tamoxifen in preventing breast cancer should be allowed to continue, despite increased estimates of the chances of women who take the drug developing endometrial cancer, the US Food and Drug Administration (FDA) was told last week.

The FDA's oncology advisory committee had been asked to judge whether new estimates of the risk of endometrial cancer meant that the trial should be stopped. It was also asked whether the conditions of eligibility for the trial should be changed.

After much discussion, the panel told the FDA that the trial should continue and that the eligibility criteria should remain unaltered. But it did call for increased monitoring of participants for endometrial cancer, while acknowledging its inability to recommend a screening method, as no one procedure is well enough understood.

The panel said that possible screening methods should be "vigorously evaluated". But it did not discuss the potential dangers of colorectal cancer, even though this may be another possible side-effect of tamoxifen.

The tamoxifen prevention trial is being run by the National Surgical Adjuvant Breast and Bowel Project (NSABP) at the University of Pittsburgh, in Pennsylvania. The aim is recruit 16,000 women at a high risk of developing breast cancer, and randomly assign them either a placebo or tamoxifen for five years. Their progress will be followed for seven years to see whether tamoxifen reduces the number of breast cancers.

Nearly 11,000 women are already taking part in the trial, and last week's meeting should have been the final word on whether or not the trial should continue. But for several reasons the trial has become mired in controversy.

First, the National Cancer Institute (NCI) suspended recruitment of participants at the end of March — along with those in all of the NSABP's other trials — following the discovery that the quality of data submitted to the NSABP had not been adequately monitored (see *Nature* 368, 679; 1994).

Most of the NSABP's other trials evaluating treatments for breast and bowel cancer have resumed recruitment of patients. But the NCI is still discussing the conditions under which recruitment to the tamoxifen prevention trial should recommence.

Few doubt that recruitment will be completed, as the National Cancer Advisory Board has already said that the trials should be reopened as soon as possible. Donald Trump, the interim executive director of the NSABP, also says that the NCI has provisionally accepted proposals for tightening up data monitoring.

But the NCI is walking on eggshells. In addition to the institute's own concerns about

the NSABP, Congressman John Dingell (Democrat, Michigan) was due to hold a hearing this week into the University of Pittsburgh's handling of allegations of scientific misconduct. And Senators Connie Mack (Republican, Florida) and Diane Feinstein (Democrat, California) have written to NCI's director, Samuel Broder, asking to be kept closely informed about the prevention trial.

Complicating matters further is a heated debate in both the United States and Britain about whether tamoxifen should be given to healthy women at all. The rationale for the prevention trial is the acknowledged fact that the drug reduces the appearance of new cancers in a large percentage of women who have already had the disease. But some question whether the potential benefit to a healthy woman of avoiding breast cancer outweighs the risks of taking a drug with serious known and suspected side-effects.

Supporters of the trial accept that the women involved should be at higher risk of developing breast cancer than other women of their age group; but they do not agree on what the increased risk should be.

The NSABP's trial recruits women from the age of 35 upwards with the same (or higher) risk of developing breast cancer as a woman of 60. During the original peer review of the trial, several scientists said that, in view of the risks involved, this 'baseline' risk had been pitched too low.

The prevention trial was approved without modifying the level of risk selected. But last week, Leslie Ford, the NCI's head of the tamoxifen prevention trials, told the FDA that, in practice, the women recruited to the trial were at a much higher risk of developing breast cancer than was called for in the trial protocol.



**Feinstein: wants to be kept informed.**

Craig Henderson, chief of medical oncology at the Mount Zion Medical Center in San Francisco, California, expressed concern at the news. "If the trial shows a benefit, and we recommend tamoxifen as a preventative, it will be given to women with the lower increased risk of breast cancer, [even though] the trial will not have shown the benefit to these women," he said.

Trevor Powles, from the Royal Marsden Hospital in London, gave the FDA panel details of a small trial being carried out to evaluate a promising endometrial screening procedure to catch changes to the endometrium early enough to prevent cancer developing. The women undergoing screening are all post-menopausal, and are members of a pilot tamoxifen prevention trial at the Royal Marsden. Each has at least a 3.8-fold higher risk of contracting breast cancer than other women of the same age.

The possible link between tamoxifen and colorectal cancer is being studied in Britain by the Medical Research Council's Toxicology Unit. The study has been prompted by unpublished findings from a Swedish group, headed by Larferic Rutqvist at the Karolinska Institute in Stockholm suggesting a possible doubling of colorectal cancer among patients taking tamoxifen.

Meanwhile, Britain's Imperial Cancer Research Fund (ICRF) recently began to recruit 15,000 women of 35 and over for a randomized tamoxifen prevention trial. The ICRF is recruiting women whose chances of developing breast cancer are twice as high as other women of the same age — a similar risk level as that in the NSABP's trial. At present, gynaecological examinations will be used to detect endometrial cancer.

The ICRF hopes for 150 fewer cases of breast cancer among the women taking tamoxifen, resulting in 50 fewer deaths. It also anticipates 40 extra cases of endometrial cancer among those taking the drug, with six extra deaths. But the Medical Research Council still insists that it will fund a trial only in women over 40 with a four-fold higher risk of developing breast cancer than other women their own age.

**Helen Gavaghan**

## Japan signs co-operation agreement with UK

Tokyo. Japan and the United Kingdom this week signed their first formal inter-governmental agreement on science and technology. The agreement is expected to boost the already substantial research links between the two countries.

The agreement was signed by William Waldegrave, the UK minister for public service and science, and Mikio Omi, Japan's minister of state for science and technology, and is a product of a visit to

Japan last year by Waldegrave and Prime Minister John Major (see *Nature* 365, 480; 1993).

It establishes a formal framework for regular high level meetings to encourage and steer collaborative research, and is partly a response to moves by other advanced nations — such as the United States, Germany and France — which have established bilateral agreements with Japan in recent years. David Swinbanks

## Why use BST?

SIR — According to a recent News story (*Nature* 368, 384; 1994), Monsanto Company has sued two dairies that characterized their products as coming from cows not treated with bovine somatotropin (BST), claiming that milk from such animals is compositionally indistinguishable from the milk of BST-treated cows. Presumably the BST does not show up in the milk.

But milk is complex, and it is virtually impossible to confirm that trace components are unaffected by BST treatment. Immunologically active steroids and proteins (such as TGF $\beta$ ) are present at low but biologically significant concentrations. Several dozen biologically active components have been identified. Even if these agents are found to be unaffected by BST, there remain factors that are not yet characterized or possibly even discovered.

For example, a few years ago we stumbled upon an immunosuppressive factor in human and bovine colostrum (not in later milk) with profound effects on cellular immune responses *in vitro*, but that cannot be accounted for by known agents. This factor is at present only partially characterized but can be detected by bioassays.

There is no difficulty, in advanced countries, in producing enough milk by the conventional method. In fact, our ability to produce far outstrips our ability to consume. And the argument that recombinant BST can lower the price of the end-product is unproven and unlikely. Most of the pennies saved by the producer will have to be paid to the masters of the technology. Many important problems cry out for technological solutions. But if a new technology isn't necessary, and we can't measure the outcome, why do it?

**Verner Paetkau**

Department of Biochemistry  
University of Alberta,  
Edmonton, Alberta,  
Canada T6G 2H7

## Quis custodiet?

SIR — Fernando Aiuti recently described the impediments to the appointment of full professors in Italy (*Nature* 367, 590; 1994). However, one of the most important faults in Italian academic institutions could well explain the failure of competitions (*concorsi*): who controls whom?

Contrary to the procedure in other countries, once a professor has been appointed (won his *cattedra*) there is no control of his scientific activity and publications, so professors can stop their research work and publications without risking their appointment. They become 'untouchable' and during their tenure (un-

til the age of 70 to 72) their scientific expertise may well fall below that of the future candidates. These professors cannot be expected to be sufficiently knowledgeable to select candidates on the basis of the best current scientific knowledge. When such a professor is in turn appointed to the *concorso* examining committee, he will be less willing to accept the candidate's international scientific credentials. So a vicious circle begins through which the lack of scientific credentials in the examining committees leads to the appointment of more poorly qualified professors.

One example is the 1989 *concorso* for full professor in pulmonary disease. Incredibly, this competition has now been going on for five years. Of the five members of the examining committee, only those with the best scientific credentials are able to appreciate the best candidates, while less qualified members tend to use the 'old boy' network. The candidates unfairly excluded have applied to the courts for justice but, although the competition was declared invalid by the Corte dei Conti (public affairs control court), the examining committee of that *concorso* stays the same. The Ministry of Universities so far has not been able to replace the committee of 'untouchables' or guarantee the teaching of pneumology in the universities that had originally asked for a professor in this subject. Who controls whom?

**Tommaso Todisco**

Pulmonary Unit,  
R. Silvestrini Hospital,  
06132 Perugia,  
Italy

## Nuclear weapons

SIR — The US refusal to export highly enriched uranium to Germany should be seen as a welcome contribution to non-proliferation (*Nature* 369, 85; 1994). In view of the stringency with which this policy is being pursued, it is surprising that the United States has not taken any action on THORP in the United Kingdom, which will provide weapons-usable plutonium to Germany, among others, and is looking to supply mixed oxide (MOX) fuel as well.

You say the material will be subject to whatever safeguards are needed to ensure that it is not diverted to military uses. But safeguards have proved inadequate even in 'law-abiding' countries such as Japan, where it was recently disclosed that 70 kg of plutonium are unaccounted for, presumed stuck in process equipment.

The sooner the close links between nuclear power and nuclear weapons are acknowledged the better. The 'inalienable right' to nuclear technology granted under the Non-Proliferation Treaty (NPT) has

been, and is being, exploited for less than peaceful purposes by countries that many would rather not see having a nuclear capability.

As far as the Campaign for Nuclear Disarmament (CND) is aware, the question of access to 'peaceful' nuclear technology is not the foremost concern of the non-nuclear NPT signatory states. On the contrary, their principal objections to the NPT as it stands are that the nuclear states have neglected their side of the bargain, that is, to pursue disarmament in good faith.

It is for this reason that CND advocates only a limited extension of the NPT, conditional on moves towards a global treaty banning all nuclear weapons. Part of CND's agenda, summed up in its *Blueprint for a Nuclear-Weapon-Free World* is a call for the establishment of an international fund to investigate, promote and invest in sustainable alternatives to nuclear power.

The only way to ensure that nuclear technology is not misused is to replace it, rather as the United States is trying to do with highly enriched uranium in Germany.

**Janet Bloomfield**

(Chair)  
Campaign for Nuclear Disarmament,  
162 Holloway Road,  
London N7 8DQ, UK

## Rough justice

SIR — It is open to anyone to write an anonymous letter accusing a scientist of faking his or her data. But one would not expect a reputable publication to print the accusation in the absence of evidence to support the claim.

In the case of Dr Justine Sergent, the *Montreal Gazette* did not feel it necessary to operate under this constraint. Neither, apparently, did McGill University find it necessary to demand that the *Gazette* publish an immediate apology (*Nature* 369, 176; 1994).

Those of us who were Justine's friends find the accusation against her unbelievable; but whatever the outcome of the inquiries currently in progress, academic justice, like every other variety, should surely rest on the principle of innocent until proved guilty. The evidence presented to all the McGill inquiries must be made public as soon as possible; the ethical standards of more people than Justine Sergent are at issue.

**John C. Marshall**

**Jennifer M. Gurd**  
Neuropsychology Unit,  
University Department of Clinical  
Neurology,  
Radcliffe Infirmary,  
Woodstock Road,  
Oxford OX2 6HE, UK



# Cocktail party effect made tolerable

**Disentangling meaningful signals from a background of otherwise distracting noise is a commonly practised skill, now shown to be mathematically feasible and even a basis for building neural networks to do the job.**

THE 'cocktail party effect' is an all too familiar problem. Put 50 people in a small room, put drinks in their hands and then observe their difficulties in communicating with each other. As the general hubbub increases, people have to raise their voices further in the hope that their neighbours will hear what they are saying. In hardly any time at all, everybody will be shouting. The hubbub rises. Communication becomes virtually impossible. In the communications community, the phenomenon is generally taken to be a kind of parable of how, in the real world, communication is perpetually babelized. The signals are buried by noise.

But those who enjoy cocktail parties usually return from them with a sense of having been engaged in conversations they have found enjoyable or otherwise instructive, however crowded the occasion may have been, which prompts a second reason why the phenomenon is so often referred to in the communications textbooks: it is a proof that intelligent beings can indeed distinguish the signals in which they have an interest from otherwise intolerable noise. Indeed, the contents of the head appear to be remarkably skilled at doing just that. But how is it accomplished?

Two theoreticians from the Institut für Theoretische Physik at Kiel in Germany, L. Molgedey and H. G. Schuster, have now produced what may be an explanation — and the specification of a neural network to go with it (*Phys. Rev. Lett.* **72**, 3634–3637; 1994). More accurately, they have built on a treatment by John Hopfield of California Institute of Technology of a seemingly different but essentially similar problem — that of how slugs process olfactory cues (smells) from distinct odorants and mixtures thereof (*Proc. natn. Acad. Sci. U.S.A.* **88**, 6462–6466; 1991).

The use of the word 'explanation' in this context, as in other matters of artificial intelligence, demands caution. To set up a mathematical model of how an intellectual task may be accomplished does not constitute a proof that the brain functions in that way. Even the elegant account by the late David Marr, more than a decade ago, of how stereopsis may be accomplished is no more than an equivalent of what the mathematicians would call an 'existence theorem'. But there are great benefits in knowing that a computational job of which the brain, by demonstration, is evidently capable can indeed also be accomplished mathematically. Being able to build a neural network from silicon is evidently a further boon.

Indeed, Molgedey and Schuster would generalize the applicability of the problem they set out to solve. Separating distinct radio signals from what is still quaintly called the aether, discriminating distinct odours from within a mixture of odorous stimulation and allocating to distinct sources within the head the jumble of electromagnetic signals typically recorded by devices attached to the exterior of the skull all come within their purview. More generally, they even suggest that their treatment may even be relevant to the way in which the brain can deal separately with distinct objects in, say, the visual field. But the cocktail party effect (which Molgedey and Schuster do not refer to in as many words) is a useful place at which to start.

So how can the sounds of different people's voices at a cocktail party be disentangled? Formally it is straightforward. Suppose there are as many receivers (pairs of ears) as there are sources of speech, which is by definition true of any real cocktail party. Suppose also that the signal picked up by each receiver is some linear combination of all the output from all the sources (voices). Then the signal  $I_i(t)$  at receiver  $i$  will be  $\sum_j C_{ij} a_j(t)$ , where the index  $j$  refers to one of the different sources,  $a_j(t)$  is its time-dependent output signal and the quantities  $C_{ij}$  are numbers. So the problem seems to be neatly solved. There are as many linear equations as there are sources (speakers); by pooling all the receivers' signals, it should be possible to work out exactly who said what by solving them (or by inverting the underlying matrix).

The trouble, at a real cocktail party, is that the quantities  $C_{ij}$  are not fixed (people move about) and that the outputs from the various sources are for all practical purposes arbitrary; people say what comes into their heads, or even lapse into silence (perhaps less often than would be prudent). More than that, there is no prospect of using the pooled information in people's heads in real time, or even quickly enough for them to be able to hold a conversation. The objective must be to arrange things so that each receiver can detect the output from just one source, no more and no fewer. How can that be done?

The starting point must be the simple truth that outputs from different sources are uncorrelated in time (on the assumption that the cocktail party has not degenerated into community singing), but that there will be a degree of auto-correlation in time in each source signal. Molgedey and Schuster sim-

ply suppose that  $\langle a_j(t) a_j(t') \rangle$ , where the angle-brackets indicate a time average, is some function of the absolute time-difference ( $t-t'$ ). There is an arithmetical difficulty in that the matrix of the quantities  $C_{ij}$  is not in general symmetrical, but the essence of the solution is to use time-correlated measurements of the receivers' signals as a means of subtracting from them quantities that yield a signal corresponding to that of just one of the sources.

With only two sources (and two receivers), it is a simple business. By definition,  $I_i(t) = C_{i1} a_1(t) + C_{i2} a_2(t)$ , so that if the objective is to make  $I_i(t)$  a faithful representation of  $a_1(t)$ , it is simply necessary to correct it by subtracting  $C_{i2} a_2(t)$ , which can surely be determined by the measurement of all possible signals and their correlation coefficients with each other. That turns out to be an arithmetically well-determined problem; there is indeed enough information to fix the coefficients, and in real time, provided they do not vary more rapidly than the definition of the problem specifies.

In reality, with more than two people at a cocktail party, the computation is a little more complicated. Even though the different sources are uncorrelated, the receivers' signals (in general made up of mixed input from all sources) will be correlated with each other. The trick is to compute corrections to these output signals  $I_i(t)$  that will reduce them to direct measures of the speech signals  $a_j(t)$ , using only information that can be gathered from the receivers themselves. The raw material must consist of measurements such as  $\langle I_i(t) I_j(t) \rangle$  and  $\langle I_i(t) I_j(t+\tau) \rangle$ , where  $\tau$  is a small increment of time; the quantities to be computed are the coefficients  $C_{ij}$  and the relative intensities of the sources and their auto-correlation coefficients.

On the face of things, it looks as if the job can be done. Indeed, Molgedey and Schuster go so far as to mix together two library records of crying babies and show that the separate sounds can be successfully disentangled from the mixed signal by a straightforward application of their technique. No doubt the next step will be to build the appropriate silicon chip to see whether it will function as intended. Patents have no doubt already been applied for, while the authors also argue that their technique should be applicable even to nonlinear mixing of the signals. It may not be long before those who habitually give noisy cocktail parties will feel bound to equip their guests with an appropriately designed headset.

John Maddox

# Drowned trees record dry spells

F. Alayne Street-Perrott

VIKING farmsteads in Greenland fjords, sunny vineyards in central England and flourishing fields of wheat and barley in south Iceland: these are some of the images conjured up by the Mediaeval Warm Epoch, as described by H. H. Lamb in *Climate, History and the Modern World*<sup>1</sup>. This period, from the tenth to the early fourteenth centuries, saw a great expansion of European civilization, in the context of persistently fine summers, matched only by the warm years of the late

droughts in the approximate intervals AD 892 to 1112 and AD 1209 to 1350; Stine's approach does not resolve interannual or decadal fluctuations within these intervals. To judge from how far the water levels dropped, the droughts were of far greater severity and duration than even the 'Dust Bowl' period between 1928 and 1934, or the recent Californian drought of 1987 to 1992. The existence of two discrete drought episodes, separated by a very wet interval, is in line with the evidence for a

The tenth to fourteenth centuries saw dramatic changes in the civilizations of the Americas, which have been attributed by some archaeologists to fluctuations in rainfall — for example, the collapse of Tiwanaku, the highest urban centre in the New World, between AD 1000 and 1100 (ref. 7); the brief florescence of Tula, in semi-arid Mexico, between AD 950 and 1150 (ref. 8); and the dramatic decline of the Anasazi cliff-dwellers in the American southwest around AD 1300 (ref. 9).

Nor was the New World the only area outside Europe to be affected by fluctuations in rainfall during mediaeval times. For example, widespread desiccation of lakes in East China occurred between AD 950 and 1250 (ref. 10). Close scrutiny of the exceptionally detailed historical records from different parts of China, however, confirms that secular variations in precipitation were complex, time-transgressive and of opposite sign in different regions<sup>10–12</sup>. Detailed, year-by-year mapping is essential if the underlying changes in atmospheric circulation are to be elucidated.

Five main causes of decadal to century-scale climatic variability are commonly identified<sup>13,14</sup>: random atmospheric variability; inherent or forced variations in the production rate of North Atlantic Deep Water; solar variability; variations in volcanic aerosols; and natural changes in the concentration of atmospheric trace gases. Whereas the last three have received considerable attention as part of the effort to predict future climate, our present lack of understanding of the natural variability of the atmospheric and oceanic circulations is a matter for serious concern. A detailed survey of the magnitude and distribution of climatic anomalies during historical warm and cold periods would be an invaluable step in this direction. □

F. Alayne Street-Perrott is in the Environmental Change Unit and the School of Geography, Mansfield Road, Oxford OX1 3TB, UK.



'Graveyard' of pine stumps along the West Walker River, testament to ancient droughts.

twentieth century. Not surprisingly, it has frequently attracted attention from climate historians as a possible analogue for the impact of future greenhouse warming<sup>2</sup>. Doubts remain, however, about the magnitude and geographical extent of mediaeval warming, especially in view of the skimpiness, ambiguity and anecdotal character of the existing documentary record, much of it in the form of Norse sagas or early monastic records<sup>1,3</sup>.

The case for global significance is strengthened by new scientific evidence presented by Scott Stine on page 546 of this issue<sup>4</sup>. Like the one in the photograph shown here, many lakes, swamps and rivers in California are bordered by drowned tree stumps, indicating that lower water levels in the past had been succeeded by flooding. What Stine does is to make a radiocarbon assay of the outer (youngest) tree rings in order to date the time of inundation, and convert these ages to calendar years using the latest correction curve for radiocarbon dates<sup>5</sup>. He then determines the duration of the preceding dry interval by counting the rings in the interior of each stump.

The samples fall into two clearly separated groups, recording sustained

significant cold snap in northern Fennoscandia during the early twelfth century<sup>3</sup>.

The four Californian sites are all vulnerable to variations in winter precipitation from Pacific storms travelling in the mid-latitude westerlies. Drought in this area is most readily attributable either to a poleward contraction of the northern circumpolar vortex, or to a shift in the upper ridge–trough pattern over North America. An overlapping group of dates from two lakes in the lee of the Patagonian Andes, where a poleward shift in the main axis of the westerlies would tend to cause drought by intensifying the rainshadow effect, leads Stine to argue that the 'mediaeval' droughts involved a simultaneous contraction and/or a change in the wave pattern of the polar vortices in both hemispheres. A noteworthy effect of a change in the number or amplitude of the upper waves would be to cause climatic anomalies of opposite sign in different regions.

Stine's work provides welcome and all too rare experimental evidence for hydrological changes, which receive much less attention than temperature changes. And it serves as a salutary reminder that decadal to centennial variations in climate involve variables other than temperature<sup>6</sup>.

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# Thrombopoietin — at last

Donald Metcalf

WHEN, for decades, a vital blood-cell growth factor has been believed to exist but has resisted all efforts to characterize it, resolution of the conundrum is a cause for celebration. So let celebrations begin — this issue contains four papers<sup>1-4</sup> indicating that the frustrations concerning the platelet-regulatory factor, thrombopoietin, have at last been overcome.

Platelets (thrombocytes) are necessary for blood clotting, and when their numbers are very low a patient is at serious risk of death from catastrophic haemorrhage. Certain diseases are primarily due to platelet defects, but most patients with thrombocytopenia are those who have received chemotherapy or bone marrow transplantation as treatment for cancer or lymphoma. The slow recovery of platelet levels in such patients is a serious problem, and has lent urgency to the search for a blood growth factor able to accelerate platelet regeneration.

More than 30 years ago, evidence began to accumulate that a humoral factor was present in the plasma of patients or animals with severe thrombocytopenia, and that it could increase platelet numbers when injected into animals<sup>5</sup>. This candidate regulator was termed thrombopoietin, and it was envisaged as being able to accelerate the maturation of megakaryocytes — the blood cells from whose cytoplasm mature platelets bud off and enter the circulation. After the development of culture techniques for growing megakaryocyte colonies, it was noted that thrombocytopenic plasma could strongly stimulate the proliferation of megakaryocyte precursors. But these observations raised more questions than they resolved, in that it became unclear whether both types of response were due to the postulated thrombopoietin or whether separate regulatory factors were involved.

The attempted purification of thrombopoietin from thrombocytopenic plasma using the conventional methods of separative protein chemistry proved to be a daunting task — plasma has a very high protein content, and blood-cell regulators are typically present in only minute concentrations. The situation was not helped by the necessity to use a cumbersome *in vivo* assay based on uptake of radiolabelled isotopes into platelets. As a consequence, whereas erythropoietin (which regulates red cell formation) and the colony stimulating factors (CSFs, which control granulocyte and monocyte formation) were identified, mass-produced in recombinant form and entered clinical use, progress with thrombopoietin remained stalled.

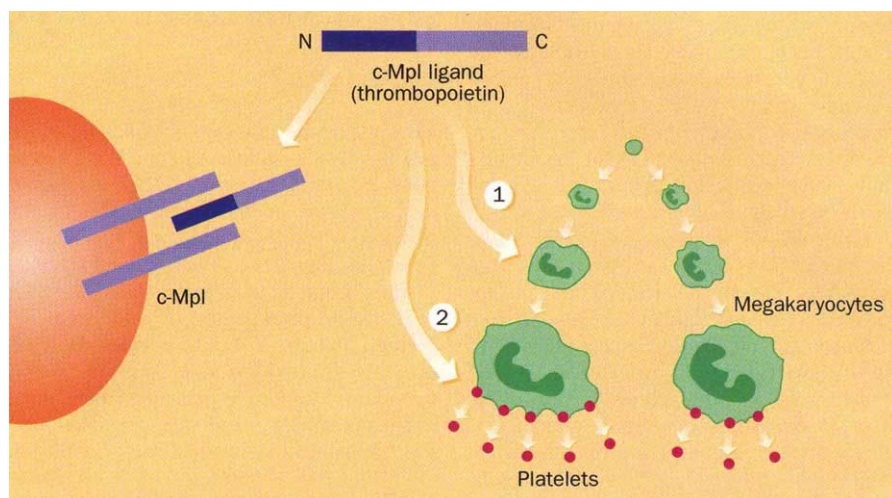
Meanwhile, four other haemopoietic regulators with proliferative actions on megakaryocytes — interleukin-3, interleukin-6, leukaemia inhibitory factor and interleukin-11 — were characterized and proved to have some ability to increase platelet levels. From the clinical viewpoint, however, the action of each is rather slow, and each has the potential disadvantage of having unwanted actions on a wide variety of other cells.

The four reports published in this issue (pages 533, 565, 568 and 571) dramatically alter things. Complementary DNAs for

did this by using *c-mpl* antisense oligodeoxynucleotides to show that these inhibited megakaryocyte colony formation *in vitro*, but had no inhibitory effects on erythroid or granulocyte-macrophage colonies.

From this point on, the story demonstrates the power of the 'receptor first' approach for detecting growth factors. As an initial step, in all four of the present studies cell lines were engineered to express the c-Mpl receptor and these cell lines provided the essential tool for monitoring and cloning the c-Mpl ligand.

de Sauvage *et al.*<sup>1</sup> achieved the heroic feat of purifying the ligand from the plasma of irradiated pigs by using c-Mpl affinity columns and, with amino-acid sequence data, isolated a porcine gen-



Thrombopoietin action. The ligand binding to the c-Mpl receptor has the capacity not only to stimulate the formation of megakaryocytes (1) but also to stimulate megakaryocyte maturation with the release of platelets to the blood (2).

both human and murine thrombopoietin have been cloned and shown to encode a glycoprotein which has apparently selective actions on megakaryocyte proliferation *in vitro*. Recombinant thrombopoietin has powerful actions in increasing platelet numbers in mice, and thrombopoietin turns out to be the main humoral regulator in situations involving thrombocytopenia.

The present success stories had an unlikely beginning. In 1990, Souyri and colleagues<sup>6</sup> established that a virus, which they had isolated as an inducer of a myeloproliferative leukaemic state in mice, had transduced in its envelope gene a cellular gene (*c-mpl*) with recognizable homology to the genes encoding an emerging family of haemopoietic growth-factor receptors. Souyri *et al.* postulated that autonomous transformation of cells by the *c-mpl* virus might be achieved by constitutive activation of this putative receptor. Last year, Methia *et al.*<sup>7</sup> demonstrated that normal c-Mpl might have special relevance as a receptor for cells of the megakaryocyte lineage; they

omized a human genomic c-Mpl ligand clone. A human genomic c-Mpl ligand clone was then isolated with the polymerase chain reaction, using porcine-based oligonucleotides, and a cDNA clone isolated from a human fetal liver library. These authors then went on to show that recombinant c-Mpl ligand increases levels of platelets when injected into animals.

Lok *et al.*<sup>2</sup> and Kaushansky *et al.*<sup>3</sup> adopted a quite different strategy. This was one based on the observations, made with a number of haemopoietic cell lines, that when autonomous transformation occurs it is often due to the acquired autocrine production by the cells of the relevant growth factor for a receptor being expressed on the cells<sup>8,9</sup>.

These workers therefore analysed clones of autonomous mutants of cells expressing the inserted candidate receptor, c-Mpl, in the hope of detecting one where transformation was based on c-Mpl ligand production. This is a chancy business, as we found to our cost using an identical approach. Of 180 autonomous clones we examined, none was producing

c-Mpl ligand and all were producing one or other CSF for which the cells normally exhibit receptors. Lok *et al.* and Kaushansky *et al.* were more fortunate, and the twenty-fourth clone tested proved to be producing c-Mpl ligand. Complementary DNA libraries were prepared from this cell line, and expression screening was undertaken using c-Mpl-bearing target cells. The upshot was isolation of a cDNA for murine c-Mpl ligand. The recombinant protein proved capable of increasing platelet levels in mice to far higher levels than those attainable with known haemopoietic regulators; and analysis *in vitro* showed that it was also able to stimulate megakaryocyte formation, particularly in combination with other factors.

Wendling and colleagues<sup>4</sup> complete the evidence that the c-Mpl ligand is indeed thrombopoietin. They show that all the megakaryocyte colony-stimulating activity and platelet-elevating activity in thrombocytopenic plasma can be removed by recombinant c-Mpl, indicating that both biological properties can be ascribed to the c-Mpl ligand.

Thrombopoietin appears to have two distinct regions separated by a potential Arg-Arg cleavage site. The amino-terminal region is highly conserved in man and mouse, and has some homology with erythropoietin and interferon- $\alpha$  and interferon- $\beta$ . The carboxy-terminal region shows wide species divergence. It is unclear so far whether the amino-terminal region has biological activity by itself. RNA analysis suggests that the liver and kidney could be major production sites of thrombopoietin. From the success of the approach using inserted *c-mpl* cDNA, it seems possible that the thrombopoietin receptor functions as a homodimer, similar to the situation with the receptors for G-CSF and erythropoietin (the latter is in part similar in sequence to c-Mpl).

The observed biological effects of thrombopoietin are in line with those of other haemopoietic regulators such as the CSFs, in that it stimulates both proliferation and maturation. Although, on present data, thrombopoietin's actions seem to be restricted to cells of the megakaryocytic lineage, it is reasonable to expect that they will probably be somewhat broader because none of the 20 or more known haemopoietic regulators is absolutely restricted in its actions to cells of a single lineage.

The impressive feature of thrombopoietin is its apparent ability to increase platelet numbers to previously unattainable levels, and the prospects look good that it will quickly find wide clinical applications (provided that there are no unforeseen side effects). But expectations do need to be realistic. A regulator needs target cells to elicit a response, and the badly damaged marrow of a patient after intensive chemotherapy may well no longer contain enough targets to allow maximum possible platelet responses. The recent clinical use of CSF-elicited peripheral blood stem cells, which themselves have an impressive capacity to

regenerate platelets<sup>10</sup>, would seem to provide a particularly suitable situation in which to use thrombopoietin.

A busy time now lies ahead for biologists and clinicians. And with at least one other group rumoured to have cloned thrombopoietin, it will also be a busy time for the lawyers arguing who has the patent rights to what should prove to be a lucrative and valuable new therapeutic agent. □

Donald Metcalf is in The Walter and Eliza Hall Institute of Medical Research, Post Office, The Royal Melbourne Hospital, Victoria 3050, Australia.

## CELL CYCLE

# p21 inhibits cyclin shock

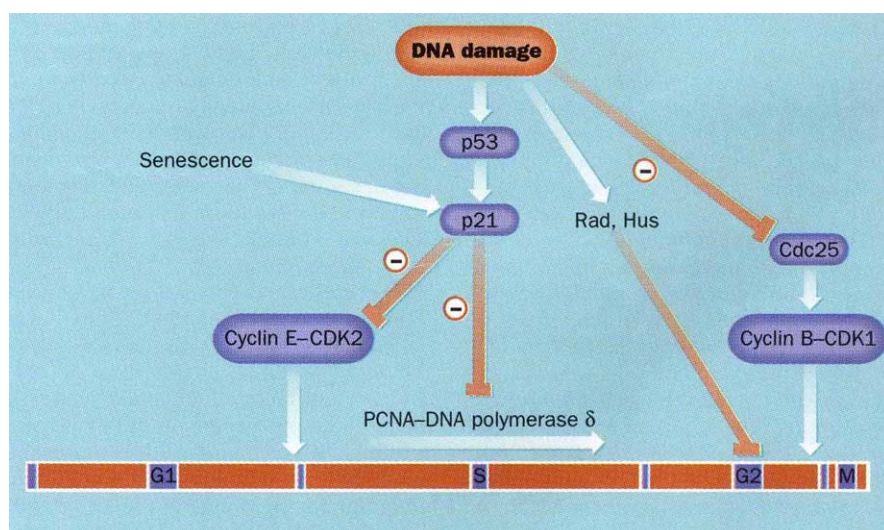
Jonathon Pines

THE cyclin-dependent kinase inhibitor p21 is only six months old and hasn't even been properly christened. But already it has emerged as a key molecule in the process whereby a cell can respond to DNA damage or senescence, and stop the protein kinases that drive the cell cycle. Now, in an unexpected development described on page 574 of this issue<sup>1</sup>, Waga *et al.* show that p21 is able to inhibit DNA replication directly, quite independently of the cyclin-dependent kinases. This finding has profound implications for understanding the regulation of both DNA synthesis and DNA repair.

The current view is that progress through the cell cycle — the alternating phases of DNA replication (S phase) and

mitosis — is regulated by a number of closely related cyclin-dependent protein kinases, the CDKs. In the past six months a host of inhibitors of the CDKs have been isolated from yeast and mammalian cells. In mammalian cells, at least four different inhibitors — p16, p21, p24 and p27 — have been identified. p16 binds only to one type of CDK, CDK4, and in April gained notoriety as a potential tumour-suppressor protein that is deleted in several tumours<sup>2,3</sup>. p24 has homology to dual-specificity protein phosphatases but its substrate is unknown. p27 blocks the cell cycle in response to transforming growth factor  $\beta$ .

But it is p21 that has attracted the most attention (and the most names). p21 was



Simplified view of the putative function of p21. p53 is activated in response to DNA damage and increases transcription of p21. If cells are in G1 phase the increased amount of p21 inhibits cyclin E-CDK2 and so prevents cells from entering S phase. If cells are in S phase, increased levels of p21 inhibit PCNA and so block DNA replication. DNA damage is also known to block the entry of cells into mitosis by preventing the activation of the cyclin B-CDK1 complex. This is achieved by stopping the activation of the Cdc25 phosphatase responsible for turning on cyclin B-CDK1, and by the activation of *rad* and *hus* genes.

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first identified as a component, along with the proliferating-cell nuclear antigen (PCNA), of almost all CDK complexes in normal but not in transformed cells<sup>4</sup>. It was subsequently isolated in a number of ways: as CIP1, a protein that binds to CDK2 in the yeast two-hybrid system; as CAP20, a protein tightly bound to CDK2 in cell lysates; as SDI1, a protein encoded by an abundant messenger RNA in senescent cells that can inhibit cell proliferation; and as WAF1, a protein that is induced by wild-type but not mutant p53 (for review see ref. 5). That last finding immediately suggested that p21 is involved in inhibiting the cell cycle in response to DNA damage, and supporting data have been quick to arrive; p21 is induced in  $\gamma$ -irradiated cells that have wild-type p53, binds to the putative S-phase promoting kinase, cyclin E-CDK2, and so prevents cells from initiating DNA replication<sup>6</sup>.

Waga *et al.*<sup>1</sup> illuminate another facet of the p21 response to DNA damage. They find that p21 will inhibit DNA replication when added into a SV40 T-antigen-dependent *in vitro* DNA replication extract, and moreover that p21 inhibits an *in vitro* DNA replication system made up of purified components. p21 seems to inhibit elongation rather than initiation events, and its inhibition can be relieved by adding excess PCNA (ironically, PCNA was once known as cyclin).

The sting in the tail is that the purified system does not contain any cyclin-CDK complexes. So p21 can inhibit the DNA-replication machinery directly, and Waga *et al.* present data to show that it does so by binding to PCNA. This provides a means by which DNA replication can be stopped in its tracks and damaged DNA repaired. Waga *et al.* point out that the DNA-repair machinery would have to be insensitive to p21 inhibition, which may pose a mechanistic problem because PCNA is required for DNA-excision repair *in vitro*, as well as DNA replication. Thus, if DNA is damaged before S phase p21 is able to prevent cells from starting DNA replication by inhibiting cyclin-CDK complexes, whereas if DNA is damaged during S phase p21 is able to halt the replication machinery by binding to PCNA (see figure).

These observations prompt a host of questions, not the least of which are how p21 is able to bind and inhibit such different proteins as the cyclin-dependent kinases and PCNA, and whether p21 is involved in DNA repair in G2 phase. It is entirely unclear how p21 inhibition is relieved after the DNA is repaired. Is p21 post-translationally inactivated, or do the levels of cyclin-CDK complexes and PCNA increase to exceed those of p21? A further possibility is that p21 is degraded in a similar fashion to the budding yeast CDK inhibitor, p40<sup>SIC1</sup>. The SIC1 protein

halts a yeast cell in a pre-S phase state by inhibiting the S-phase CDK<sup>7</sup>, and inhibition is relieved by the ubiquitin-mediated degradation of p40<sup>SIC1</sup> (K. Nasmyth, personal communication). SIC1 has a possible counterpart in fission yeast, p25<sup>rum1</sup>, which inhibits the fission yeast CDK (Cdc2) and also halts the cell in a pre-S phase state. The product of the *rum1* gene is required for the proper coordination of S phase and mitosis in the normal, non-perturbed cell cycle<sup>8</sup>. This puts mammalian p21 in a broader context.

Waga *et al.* suggest that because p21 is able to inhibit both cyclin-CDK complexes and PCNA, it may provide a link between the cell-cycle regulators and the DNA-replication machinery, along the lines of SIC1 and Rum1. But thus far p21 seems to be primarily involved in regulating DNA replication in response to adverse conditions such as DNA damage, so the mammalian homologues to Rum1 and SIC1 may have yet to be identified.

#### ASTRONOMY

## Much ado about something

Douglas Richstone

DURING the three decades since quasars were discovered, a consistent picture of their energy source has been built up — that they are black holes fed by nearly axisymmetric influxes of material, and that the gravitational energy of the accreted mass powers the emitted radiation. Where, though, was the proof that such black holes exist? At a conference earlier this month, astronomers had the chance to hear in detail how highly publicized results from the Hubble Space Telescope (HST) measured up\*.

Before the HST, there had been some evidence both for the geometrical configuration of the accretion disk in quasars and active galactic nuclei (AGNs), and for the presence of a small but massive object which might be a massive black hole in normal, quiescent galaxies that might once have been AGNs.

The Virgo elliptical galaxy M87 seemed an obvious target for observations, as it is one of our nearest active galaxies (it is admittedly rather a weak source, but qualifies as an active galaxy through its variable nuclear radio source and its well collimated jet). A lingering frustration over the past 15 years has been the inability to identify an axisymmetric structure or black hole in the galaxy, so when the superb angular resolution of the repaired Hubble Space Telescope allowed an apparent accretion disk to be resolved,

Conversely, at present there is no evidence that either of the two yeast CDK inhibitors interacts with PCNA, so there may be other yeast inhibitors with roles more analogous to p21. Intriguingly, the *plutonium* gene in *Drosophila*, which encodes an inhibitor of DNA replication, is adjacent to the PCNA gene<sup>9</sup>. All in all, CDK inhibitors look set to reach critical mass in the very near future. □

Jonathon Pines is at the Wellcome/CRC Institute, Tennis Court Road, Cambridge CB2 1QR, UK.

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the results made headline news around the world.

If axisymmetric, this object is nearly perpendicular to the observed radio jet and is about 65 light years across (see News, *Nature* **369**, 345; 1994). Spectra of the gas obtained with the Hubble indicate a central mass of about 3 billion solar masses provided it is in circular rotation, sufficient to have provided quasar luminosities by accretion in the past (H. Ford, Johns Hopkins Univ.; R. Harms, Computer Sciences Corporation).

Ford's report at the meeting met with general acceptance, although loopholes remain for the dedicated sceptics. A cluster of a billion neutron stars or white dwarfs smaller than the observed region of the disk would be small enough and dark enough to account for the observations and could survive against two-body relaxation, core collapse or stellar collisions for longer than the age of the Universe. No one, however, was willing to advocate such a model, at least in public, as its manufacture seems as daunting as a single massive black hole. There is general agreement that the identification of this rapidly rotating disk roughly perpendicular to the radio jet is a big step in the steadily solidifying picture of the quasar phenomenon as an accretion disk feeding a massive black hole.

Another key area where the HST is proving its use is the theory of evolution of massive stars. HST permits the study of individual ultraviolet bright knots in star-

\*184th Meeting of the American Astronomical Society, Minneapolis, 29 May–2 June 1994.

burst and interacting galaxies. Work on knots in two such galaxies, II Zw 40 and NGC 4861 (W. Vacca, Univ. California, Berkeley), identifies young star clusters with radii smaller than 300 light years. These dense young clusters, with masses as large as a million solar masses, may conceivably be young globular clusters. Spectroscopy reveals a large ratio of Wolf-Rayet stars to early O stars in these objects. This is a surprise: the most massive stars live for 3 to 5 million years on the main sequence as O stars, evolving off it to Wolf-Rayet stars for perhaps a tenth of that time.

The straightforward explanation for large numbers of the shorter-lived evolved species is that the timescale for star birth in these knots is short, but that the observation is well timed, so that we happen to catch the knots in this short-lived evolved state. If so, we should see the discovery of many more knots that are O-star rich as our statistical sample improves.

On the other hand, the ratio may be telling us something new and surprising about the evolution of massive stars. HST studies of the cluster R136 in the Large Magellanic Cloud provide some evidence in this direction. Once suspected of being a single supermassive star, this has been recognized for several years as a very dense cluster of young massive stars. It is now possible to exploit the HST's ultra-violet response and high resolution to obtain spectra from the individual stars in this dense cluster. A spectrum of one of them, the O3 star R136a5, yields an estimated mass-loss rate of  $2 \times 10^{-5}$  solar masses per year, about five times what was expected (S. Heap, NASA Goddard Space Flight Center). If continuous, this mass-loss rate is large enough to dominate the early evolution of the star, having even more effect than does thermonuclear burning.

Another clue that something is amiss in our understanding of the evolution of massive stars comes from Eta Carinae. Continuum images obtained with the refurbished HST confirm the bipolar nature of its outburst last century and reveal fragments of a disk that axially confined the explosion. Analysis of historical data had shown that the energy of the explosion about 100 years ago corresponds to the energy now observed in the flow, about  $10^{47}$  erg. New data, especially spectroscopy from the HST, reveal the star itself for the first time (K. Davidson, Univ. Minnesota; D. Ebbets, Ball Aerospace). It is a roughly 100-solar-mass star at about 25,000 K and with a wind of about  $3 \times 10^{-4}$  solar masses per year. This type of star seems able to produce an outburst of supernova luminosity more than once.

Theoretical developments were highlighted in two special sessions on dynamics and on supercomputing in astronomy.

The latest supercomputing efforts in hydrodynamic cosmology seem naturally to predict galaxy formation with a scale-dependent bias in a variety of models (J. Ostriker, Princeton Univ.), under reasonable but idealized treatments of small-scale physics. The bias appears as elevated contrast: dense regions have an enhanced ratio of galaxy density to dark matter density. This is worrying, as a choice of cosmological model is sometimes dictated by comparing simulations to the evolution of structure with redshift over modest look-back times. Questions remain, however, about the fidelity of simulations of large-scale structure, even in the most extensive simulations yet made (G. Lake, Univ. Washington).

In stellar structure, too, simulations of turbulence appropriate to understanding convective layers in stars fall well short of the required dynamic range, as the largest structures may be over 100,000 kilometres across and the dissipation of energy occurs

on scales of 100 metres. Nonetheless, successes in understanding rotationally constrained turbulence through increasingly realistic simulations lend some hope of resolving certain issues, for example the disparity between the rotational pattern in a convective region expected from theory and 'observed' by helioseismology (J. Toomre, Joint Institute for Laboratory Astrophysics, Colorado). And the application of two-dimensional hydrodynamics deeper in more massive stars (W. D. Arnett, Univ. Arizona) shows that chemical inhomogeneities develop in the oxygen-burning shell before core collapse, strongly affecting the nickel yield during the supernova explosion. Like the HST, supercomputing seems to be on the verge of improving our understanding of stars and the Universe. □

Douglas Richstone is in the Department of Astronomy, University of Michigan, Ann Arbor, Michigan 48109, USA.

## GEOLOGY

# The new treasure seekers

Anthony E. Fallick

For the preparation of emerald: mix together in a small jar  $\frac{1}{2}$  drachma of copper green (verdigris),  $\frac{1}{2}$  drachma of Armenian blue (chrysocola),  $\frac{1}{2}$  cup of the urine of an uncorrupted youth and  $\frac{2}{3}$  the fluid of a steer's gall. Put into this the stones, about 24 pieces weighing about  $\frac{1}{2}$  obolus each. Put the lid on the jar, seal the lid all around with clay, and heat for six hours over a gentle fire made of olive-wood... You will find that they have become emeralds.

TIMES have moved on since this recipe for gemstone enhancement appeared in the *Papyrus Graecus Holmiensis*<sup>1</sup>, but emerald formation still holds some surprises. Work by Ottaway *et al.*<sup>2</sup> (page 552) and by Giuliani and his colleagues<sup>3-5</sup> shows that a new consensus seems to be emerging for the mode of formation of Colombian emeralds which implicates the interaction of basinal fluids with evaporites and organic-rich sedimentary horizons. Because this contrasts so forcefully with the conventional granite-greenstone model for pegmatite-associated emeralds and beryls, and removes the requirement for related igneous activity, it could well have profound consequences for exploration strategies.

According to J. R. Sauer<sup>6</sup>, emeralds are the gemstone longest known to man (having been mined in Egypt about 5,000 years ago); they are much rarer than diamonds and, weight for weight, have a value thousands of times that of gold. Colombian emeralds are especially prized for the intensity of their colour, and this country has become the world's most

important emerald producer, with registered exports worth 63 million US dollars in 1987 (ref. 3); stones from the inhospitable Muzo mine area set the standard against which others are compared. Herein lies an enigma, because the Colombian emeralds are found in sedimentary rocks and are not obviously associated with igneous activity, whereas emeralds elsewhere in the world are commonly found in coarse-grained dykes (pegmatites) intimately and genetically related to magmatic processes (see, for example, ref. 7).

Emerald is the chromium- (and vanadium-) rich variety of the mineral beryl,  $\text{Be}_3\text{Al}_2\text{Si}_6\text{O}_{18}$ . Because the  $\text{Be}^{4+}$  ion is too small to substitute in most silicate lattices, it is concentrated in residual fluids associated with crustally influenced acid magmas such as granites. If

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these granite-related residual fluids then infiltrate or interact with basic rocks rich in chromium, we have the unusual combination of beryllium and chromium to produce emerald in the late stages of crystallization; this is a somewhat simplistic version of the granite-greenstone genetic concept.

But the origin of emeralds from the Colombian Eastern Cordillera is now pictured rather differently. The fluids involved have been characterized as sulphate-bearing hydrothermal brines rich in the heavy oxygen isotope  $^{18}\text{O}$  (refs 2–5, 8, 9) which were probably formed by the interaction of basinal fluids with Jurassic and Lower Cretaceous evaporites. As Ottaway *et al.*<sup>2</sup> describe, these fluids then interacted with organic-rich shales and limestones, resulting in oxidation of organic matter, thermochemical sulphate reduction and release of organically bound beryllium, chromium and vanadium. The black shales became characteristically bleached (they are referred to as *cenicero* zones — literally, 'ashtray') and pyrite, calcite and emerald precipitate together with accessory lode-filling phases. So sulphur is derived from the fluids, but carbon, beryllium, vanadium and chromium are derived from the shales in general and the organic matter in particular (see also refs 3–5, 8, 10, 11). The oxygen, carbon and sulphur stable-isotope data (for the brines, calcite and pyrite, respectively) are all in accord with this picture which thus neatly integrates field, mineralogical and geochemical aspects.

Fluid inclusion data<sup>2</sup> suggest that the temperature of homogenization and NaCl dissolution was around 330 °C. Whether this can also be considered as a trapping temperature (and so the emerald formation temperature) then depends on whether a pressure correction is required to account for the depth at which the processes occurred. The temperature would be 50 °C higher<sup>1</sup> for formation at 1 kilobar.

Cheilletz *et al.*<sup>5</sup> combined K–Ar and  $^{40}\text{Ar}$ – $^{39}\text{Ar}$  dating of mica associated with Colombian emerald deposits, and obtained concordant ages of 31.5–32.6 Myr before present for the Quipama-Muzo zone (although whether this includes the actual mine sampled by Ottaway *et al.* is not clear to me). At nearby Coscuez the age is slightly older, 35–38 Myr before present. With this latter age constraint and a subsidence model for the Eastern Cordillera, they then deduced a formation depth of around 4,500 m, corresponding to 1.1 kbar pressure. Taking this with their own fluid inclusion evidence resulted in a formation temperature estimate of 290–360 °C. So both research programmes converge on a moderate-temperature epigenetic model involving hydrothermal brines and sedimentary shales. The importance of thermochemic-

al sulphate reduction is highlighted by Ottaway *et al.* and the absolute ages provided by the French team<sup>5,12</sup> provide a critical temporal framework. Furthermore, in other areas Ottaway *et al.* suggest that the *cenicero*-like zones may have value for exploration, as the overall new model very clearly does.

What, then, of the role of "uncorrupted

youth" in the genesis of emeralds? Unfortunately, as Watson said of the Giant Rat of Sumatra, this is a story for which the world is not yet ready. □

Anthony E. Fallick is in the Isotope Geosciences Unit at the Scottish Universities Research and Reactor Centre, East Kilbride, Glasgow G75 0QU, UK.

## TRANSCRIPTION

# The essential twist

Ronny Drapkin and Danny Reinberg

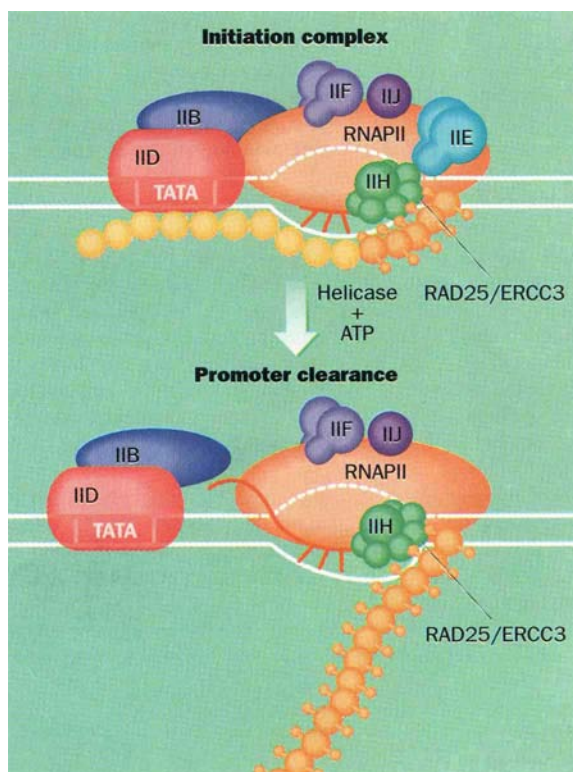
DURING initiation of gene transcription, the DNA duplex at the promoter region melts to grant RNA polymerase access to the DNA template strand. Unlike RNA polymerases I and III, transcription initiation by RNA polymerase II (RNAPII) requires hydrolysis of the ATP (or dATP)  $\beta$ - $\gamma$  bond<sup>1</sup>. Strikingly, the activity that catalyses this reaction and the precise energy-dependent step have eluded identification for years. On page 578 of this

issue<sup>2</sup>, Guzder *et al.* reveal that the yeast RAD25 DNA-repair protein possesses an ATP-dependent, DNA-unwinding activity that is essential for transcription by RNAPII. This protein, and its human counterpart ERCC3, have roused enormous interest because of their intimate connection to the basal transcription apparatus.

RNAPII requires several general factors to orchestrate transcription initiation

from protein-coding genes. Most of these factors have been purified and their corresponding complementary DNAs isolated. General transcription factor IIH (TFIIH, also called BTF2) has exhibited complexity beyond expectation. Like its yeast and rat homologues, TFIIH is a multi-subunit factor that has an intrinsic ATPase activity and a kinase specific for the carboxy-terminal domain (CTD) of the largest subunit of RNAPII (reviewed in ref. 3). Initial reports alluded to the possibility that CTD phosphorylation might account for the unique ATP requirement exhibited by RNAPII. The observations that the TFIIH kinase can also use GTP in the phosphorylation reaction, and that a form of RNAPII without a CTD can catalyse transcription *in vitro* yet still require ATP hydrolysis, suggested otherwise. Insight into the identity of the ATP-dependent activity came when the largest subunit of TFIIH was identified as being the ERCC3 DNA-repair protein<sup>4</sup>.

ERCC3 was initially characterized as the gene responsible for the DNA-repair defect in xeroderma



Model for the role of the RAD25/ERCC3 subunit of TFIIH in transcription initiation. The general transcription factors (IID, IIB, IIF, IIE, IIH and IIJ) and RNA polymerase II (RNAPII) assemble on the promoter to form the complete initiation complex. TFIIH can phosphorylate (orange) the carboxy-terminal domain (CTD) of the largest subunit of RNAPII. Before it is phosphorylated, the CTD remains in contact with the TATA-binding protein of TFIIID. Initiation appears to involve two energy-dependent steps, open complex formation and promoter clearance. The RAD25/ERCC3 subunit probably acts at the second of these two steps to catalyse promoter clearance in an ATP-dependent reaction. The RNA/DNA hybrid is in scarlet.

pigmentosum group B (reviewed in ref. 5), and it contains the canonical helicase motifs that typically enable proteins to unwind RNA and/or DNA duplexes in an ATP-dependent fashion. Indeed, ERCC3 allows TFIH to unwind DNA locally in an ATP-dependent reaction<sup>4</sup>. Guzder *et al.* now furnish direct biochemical evidence that RAD25, the yeast equivalent to ERCC3, not only contains ATP-dependent helicase activity, like ERCC3, but also that this helicase activity is vital for RNAPII-mediated transcription<sup>2</sup>.

A string of recent papers has revealed that *ERCC2*, the gene that corrects the DNA-repair defect in xeroderma pigmentosum group D (ref. 5), also encodes a component of human and yeast TFIH, and that the entire TFIH complex can function in DNA-excision repair independently of its involvement in transcription<sup>6-9</sup>. The yeast equivalent of *ERCC2* is *RAD3*, and like *RAD25* it encodes a DNA helicase required for DNA-excision repair. *RAD3* and *RAD25* are further related in that both are essential for viability in yeast.

The difference between the two proteins comes from analysis of key mutations in the nucleotide-binding domain. Such a mutation in *RAD3* abolishes its ATPase/helicase activity, impairing its DNA-repair function, but not affecting cell viability, indicating that the *RAD3* helicase is not required for the protein's essential function<sup>10</sup>. Indeed, this mutation does not affect the transcriptional role of yeast TFIH (ref. 6). A similar mutation in *RAD25*, however, is lethal, implicating the ATPase/helicase activity of this protein as the essential component<sup>9,11</sup>. Prakash and colleagues have provided compelling evidence, both *in vivo* and *in vitro*, that *RAD25* (ref. 2) and *RAD3* (ref. 12) are essential for viability because of their involvement in transcription by RNAPII. The observation by Guzder *et al.*<sup>2</sup>, that *RAD25* with a mutation in the nucleotide-binding pocket is defective in transcription, clearly shows that, unlike *RAD3*, the ATPase/helicase activity of *RAD25* is essential for its function in transcription and cell viability.

What precisely does *RAD25/ERCC3* helicase do in transcription? Open complex formation is ATP dependent<sup>13</sup>, as is the helicase activity of *RAD25/ERCC3*. So, most logically, it functions in open complex formation during transcription initiation. Interestingly, this step is not entirely specific for ATP: it appears that at high enough concentrations, any nucleotide can provide the  $\beta$ - $\gamma$  energy bond, with ATP having the lowest  $K_m$  (J. Gralla, personal communication). Because the helicase activity of *RAD25/ERCC3* is specific for ATP, it is unlikely that it catalyses open complex formation.

What else might the *RAD25/ERCC3* helicase do? Studies on the composition of

the RNAPII ternary complex have excluded the involvement of *RAD3/ERCC2*, *RAD25/ERCC3* and TFIH in elongation, as none of these factors has been detected in the ternary complex (P. Kumar, L. Zawel and D. Reinberg, unpublished results). TFIH and ATP may act at a stage subsequent to open complex formation yet before elongation<sup>14</sup>, a phase called promoter clearance. This phase is akin to a functional roadblock, bypass of which requires energy in the form of ATP. Once the polymerase forges through this roadblock, the TFIH helicase activity (provided by *RAD25/ERCC3*) and the ATP cofactor are no longer required. Moreover, the use of a pre-melted linear template (a template already in the open conformation) that extends over the transcription start site circumvents the requirement for ATP in RNAPII transcription<sup>15</sup>. This bypass probably encompasses the open complex and promoter clearance phases of initiation and obviates the requirement for the TFIH (*RAD25/ERCC3*) helicase.

This study may also shed light on the observation that superhelicity of the DNA template circumvents the requirement for TFIH and ATP hydrolysis<sup>16,17</sup>. Superhelical tension perhaps provides the energy for a stable open complex, similar to the pre-melted heteroduplex, thereby bypassing the ATPase requirements for open complex and promoter clearance.

On current understanding, it seems that open complex formation and promoter clearance are two energy-dependent steps. It is likely that the *RAD25/ERCC3* subunit of TFIH functions at the second step, but the activity involved in open complex formation remains unknown. We will have the answer, in mechanistic terms, only after nucleotide and factor requirements are biochemically correlated. It should not be long in coming. □

Ronny Drapkin and Danny Reinberg are in the Department of Biochemistry, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854, USA.

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## Lagging ahead

THE gas butadiene is an ingredient of synthetic rubber. It has a nasty tendency to polymerize prematurely. The interior of a pipe or tank of the gas often becomes coated with a mass of frothy polymeric solid. Once initiated, this 'popcorn polymer' grows at the expense of the gas. It can jam valves, block pipes and even burst containers. The higher the temperature, the more luxuriantly it grows. The infestation has to be cleared away mechanically, and the installation 'disinfected'.

Daedalus sees opportunities in this nuisance. He points out that, like foamed polystyrene, butadiene popcorn polymer is an excellent heat insulator. Imagine, he says, a boiler room or industrial plant with a wide range of complex hot vessels and pipes. Conventional lagging would be expensive to apply, and tricky to manoeuvre into all the little corners. But release butadiene into the room, and it would polymerize spontaneously to popcorn polymer on all the hot parts. It would lag the whole installation, including all the tricky and inaccessible details. Furthermore, the process would be self-optimizing. A very hot object would acquire a thicker coating before its lagged surface was cool enough not to attract further polymerization. Every part would thus receive just enough insulation for its temperature.

DREADCO's chemists are now exploring the popcorn reaction. They argue that a reaction so hard to prevent must be very easy to encourage. By adding copolymers and modifiers, they hope to perfect an autolagging vapour whose strongly temperature-dependent polymerization gives an ideal foamy or whiskery thermal insulator. Released among the steam pipes and heat exchangers of chemical works and generating stations, 'Vapourlag' will coat their most complex recesses with perfectly proportioned insulation. Blown under the floorboards of a house, it will insulate the longest and most labyrinthine central-heating and hot-water pipes. Expensive, labour-intensive lagging will be a thing of the past.

The converse problem, that of insulating cold surfaces, defeats this elegant chemistry. But Daedalus recalls that  $\alpha$ -methylstyrene does not polymerize above 0 °C; for a long time it was thought not to polymerize at all. The DREADCO team hopes to devise an analogous Vapourlag monomer which can only polymerize on cold surfaces. The world's deepfreezes and refrigerators, and its more weather-beaten houses, could then also gain the benefits of cheap, easily renewed Vapourlagging.

David Jones



# fMRI of human visual cortex

**SIR**—The primate visual system contains many distinct visual areas, each with its own rich structure<sup>1</sup>. Few detailed measurements of these areas have been made in humans, however, because of the poor spatial resolution of functional neuroimaging methods. For example, the spatial resolution in positron emission tomography (PET) studies<sup>2</sup> is typically 7–10 mm. Using functional magnetic resonance imaging (fMRI), we made detailed measurements of the topography of human primary visual cortex. Our experiments were performed using a conventional clinical scanner, and lasted only a few minutes. We measured reliable differences in activity between cortical locations separated by less than 1.4 mm along continuous strips of visual cortex. The method should have broad clinical and scientific applications.

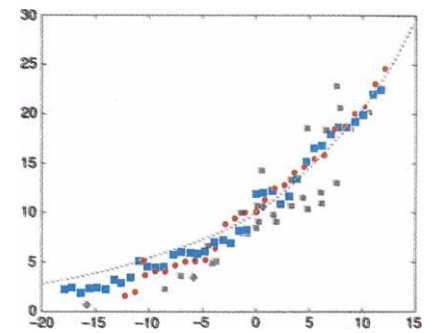
The receptive fields of neurons in human primary visual cortex follow a precisely organized retinotopic map: as a stimulus moves from the fovea to the periphery the locus of responding neurons varies from posterior to anterior portions of the calcarine sulcus, the site of human primary visual cortex<sup>3</sup>. Hence, we can use the periodic stimulus illustrated in Fig. 1a to generate waves of neural activity that travel the length of the calcarine sulcus.

As the stimulus slowly expands, each visual field location alternates between the uniform field and checkerboard. Importantly, the timecourse of the alternation depends upon visual field location; peripheral locations are delayed relative to foveal locations. When subjects view this stimulus, neural activity in the anterior portions of the calcarine should be delayed relative to activity in the posterior calcarine.

Subjects viewed four cycles of this stimulus during a 192-second session, while fMRI measurements of neural activation were continuously acquired. The fMRI signal that we measured depends upon venous blood oxygenation<sup>4,5</sup>. Figure 1b plots the time-varying fMRI signal measured at points along the calcarine sulcus. The measured activation oscillated with the stimulus period of 48 s, and the signal was delayed systematically along the sulcus, as expected.

To evaluate the quality of our measurements, we estimated the distance in cortex over which we could obtain reliable differences in the timecourse of the fMRI signal. To do this, we found the best-fitting (least-squares) sinusoid at the stimulus frequency for each of the four stimulus cycles at each measured cortical location. The mean of the phase-delays of these four sinusoids estimates the signal delay at that location, and the standard deviation of these phase-delays quantifies the precision of our measurements. If we define reliable separation as two standard errors, then the fMRI signal distinguished activation at points in cortex separated by 1.35 mm, on average.

We used this paradigm to measure the cortical representation of the central 24 degrees of the visual field. We tested two subjects with a 24-degree stimulus, and analysed our data along regions of interest approximately 30 mm in length. We converted delay in activation to retinal eccentricity by assuming that the maximal observed delay equalled the maximal stimulus eccentricity. Figure 2 shows our results, together with estimates of the retinotopic organization of human primary visual cortex based on direct electrical stimulation<sup>6</sup>, PET<sup>2</sup> and focal lesions in



**FIG. 2** Measurements of human V1 retinotopic organization. Cortical distance is measured relative to the location responding best to a stimulus at 10° of retinal eccentricity. The red circles and blue squares are two subjects from the present study. Grey squares are from a microstimulation study on a single observer<sup>4</sup>; diamonds, pooled data from 5 observers in a PET study<sup>5</sup>; dashed line estimated using the locations of scotoma in stroke patients and single-cell data from non-human primates<sup>9</sup>.

stroke patients<sup>7</sup>. Note the superior density and regularity of the fMRI results, which were obtained within a brief experimental session.

The method of measuring cortical properties by temporal correlation with a periodic stimulus has wide applicability<sup>8</sup>. It can be used without modification to map the retinotopic structure of other visual areas in human cortex. In addition, it should be possible to adapt our technique to measure response selectivity for properties such as colour and motion, and to measure nonvisual cortical maps. Finally, the ability to map brain areas quickly using a conventional MRI scanner should be useful clinically for diagnosis, surgical planning and studies of recovery of function.

**Stephen A. Engel**

**David E. Rumelhart**

**Brian A. Wandell**

*Department of Psychology,*

**Adrian T. Lee**

**Gary H. Glover**

*Department of Radiology,*

**Eduardo-Jose Chichilnisky**

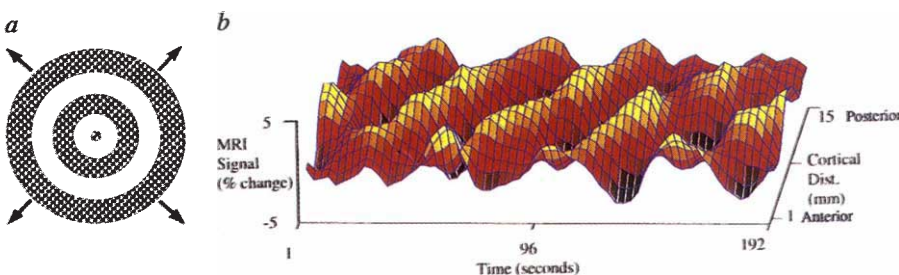
*Neuroscience Program,*

**Michael N. Shadlen**

*Department of Neurobiology,*

*Stanford University, Stanford,*

*California 94305, USA*



**FIG. 1** Mapping receptive field locations along the calcarine sulcus. The observer viewed two concentric expanding annuli presented on a grey background. Each annulus contained a high-contrast flickering (8.3 Hz) radial checkerboard pattern. The outer diameter of an annulus was bounded at 10°; as the larger annulus disappeared, a new annulus grew in the centre of the display. The image sequence had a period of 48 s and was repeated four times in a single experiment. We acquired activation images every 1.5 s in the plane of the calcarine while subjects viewed four cycles of the stimulus over a 192-s interval. We used a clinical scanner with a  $T_2^*$  sensitive gradient recalled echo pulse sequence with spiral readout<sup>9</sup>. The data had an in-plane voxel size of 1.03 mm<sup>2</sup>, a through-plane resolution of 5 mm, and were interpolated to a pixel size of 0.78 mm<sup>2</sup> to fit a 256 × 256 grid. **a**, Stimulus pattern at a single moment of time. **b**, Timecourse of activation of individual pixels located along the length of the calcarine sulcus. For display purposes only, the activation data were first smoothed with a Gaussian and then every other point was plotted.

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# Origin of introns — early or late?

SIR — Kersanach *et al.*<sup>1</sup> assert that the existence of five spliceosomal introns in identical positions in nuclear genes encoding glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) of eubacterial (chloroplast *GapA/B*) and uncertain (cytosolic *GapC*) ancestry provides “strong evidence in favour of the ‘introns-early’ hypothesis” for the origin of spliceosomal introns, and thus “lends strong support to the exon theory of genes”. Two of these five identical intron positions were already known in 1988 (refs 2, 3) and led us to comment on their evolutionary significance in 1991 (ref. 4). Despite the new data, we still consider that all of these shared positions represent “parallel insertion of different introns”<sup>4</sup>. Our rationale, part of which we elaborate below, is fourfold: (1) these five GAPDH

introns have a very limited phylogenetic distribution, as do most of the numerous introns in this gene; (2) there are likely to be preferred sites for intron insertion (the proto-splice site<sup>5</sup>, which is evident at four of the shared GAPDH intron positions), and thus the likelihood of parallel insertion is probably much higher than Kersanach *et al.*<sup>1</sup> calculate; (3) many of the putatively ancient GAPDH exons (averaging only 7.2 codons in a hypothetical ancestral GAPDH gene containing all 47 known introns) as well as some present-day GAPDH exons (as small as five nucleotides!) are in our view too tiny to be useful in ancient gene assembly as posited by the exon theory of genes<sup>6</sup>; (4) common ancestry would require the relatively recent, massive and coincidental loss of introns from many eubacterial lineages.

Kersanach *et al.*<sup>1</sup> in their Fig. 1 fail to include numerous GAPDH genes that do not contain introns, including all of the many sequenced protistan and eubacterial genes. The protistan lineages are critical

to our understanding of eukaryotic diversity; in particular, *Giardia* and *Trypanosoma* represent deeply branching lineages for which no spliceosomal introns have been reported (ref. 4, and our unpublished data). When all examined GAPDH genes are considered (see figure), it becomes apparent that each of the five ‘shared’ introns is present only in two highly restricted and distantly related lineages. Thus, the possibility must be considered that these introns arose independently and recently in each specific lineage, as opposed to being homologous and ancient, and lost pervasively throughout eukaryotes and prokaryotes. In fact, virtually all the 47 known GAPDH introns are very limited in their phylogenetic distribution (see Fig. 1 of ref. 1).

Acceptance of these five ‘shared’ introns as homologous and ancient forces one to postulate their retention for much or all of eubacterial evolution before the endosymbiotic origin of plastids, as well as their subsequent independent loss from many eubacterial lineages in parallel<sup>4</sup>. By extension, this ‘introns-early’ view requires that the estimated thousands of other introns in nuclear genes of eubacterial ancestry survived for most of eubacterial history only to be extinguished recently and completely from all eubacteria. We believe a more plausible explanation is that nuclear genes of plastid and mitochondrial ancestry acquired all their introns subsequent to their transfer from the organelles to the nucleus. Consistent with this proposal, indeed indicative of a post-endosymbiotic origin of all spliceosomal introns, we find lineage-specific covariance between intron numbers in nuclear genes of eukaryotic and organellar/eubacterial ancestry. In conclusion, the data in ref. 1 are best explained by the late, lineage-specific acquisition of introns rather than primordial intron retention. The introns in question are not, in our view, homologous, but instead are the result of recent, independent parallel insertion into conserved target sites.

**John M. Logsdon Jr**

**Jeffrey D. Palmer**

Department of Biology,  
Indiana University,  
Bloomington,  
Indiana 47405, USA

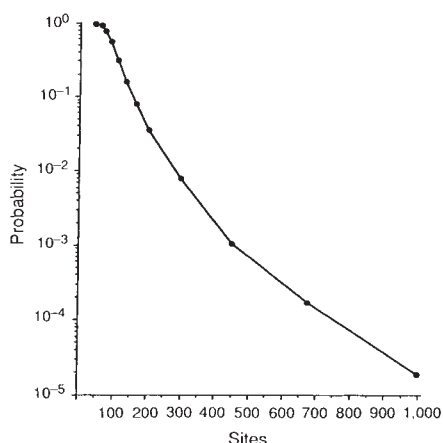
|                                | 25 | 28 | 29 | 30 | 46 |                                 | 25 | 28 | 29 | 30 | 46 |
|--------------------------------|----|----|----|----|----|---------------------------------|----|----|----|----|----|
| <i>Zea GapC1</i>               | +  | —  | +  | —  | —  | <i>Thermotoga</i>               | —  | —  | —  | —  | ?  |
| <i>Zea GapC4</i>               | +  | —  | +  | —  | +  | <i>Thermus</i>                  | —  | —  | —  | —  | —  |
| <i>Pisum GapC1</i>             | +  | —  | +  | —  | +  | <i>Bacillus c</i>               | —  | —  | —  | —  | —  |
| <i>Arabidopsis GapC</i>        | +  | —  | +  | —  | —  | <i>Bacillus m</i>               | —  | —  | —  | —  | —  |
| <i>Chlamydomonas GapC</i>      | —  | —  | +  | —  | —  | <i>Bacillus s</i>               | —  | —  | —  | —  | —  |
| <i>Chondrus GapC</i>           | —  | —  | —  | —  | —  | <i>Corynebacterium</i>          | —  | —  | —  | —  | —  |
| <i>Gracilaria GapC</i>         | —  | —  | —  | —  | —  | <i>Clostridium</i>              | —  | —  | —  | —  | —  |
|                                |    |    |    |    |    | <i>Streptococcus</i>            | —  | —  | —  | —  | —  |
| <i>Homo</i>                    | —  | +  | —  | —  | —  | <i>Rhodobacter</i>              | —  | —  | —  | —  | —  |
| <i>Gallus</i>                  | —  | +  | —  | —  | —  | <i>Zymomonas</i>                | —  | —  | —  | —  | —  |
| <i>Drosophila m Gap1,2</i>     | —  | —  | —  | —  | —  | <i>Pseudomonas</i>              | —  | —  | —  | —  | —  |
| <i>Drosophila h</i>            | —  | —  | —  | —  | —  | <i>Escherichia c gapA</i>       | —  | —  | —  | —  | —  |
| <i>Schistosoma</i>             | —  | —  | —  | —  | —  | <i>Escherichia c gapA (13)</i>  | —  | —  | —  | —  | ?  |
| <i>Caenorhabditis e gpd1,4</i> | —  | —  | —  | +  | —  | <i>Escherichia b gapA (3)</i>   | —  | —  | —  | —  | ?  |
| <i>Caenorhabditis e gpd2,3</i> | —  | —  | —  | —  | —  | <i>Escherichia h gapA (2)</i>   | —  | —  | —  | —  | ?  |
| <i>Caenorhabditis b gpd2,3</i> | —  | —  | —  | —  | —  | <i>Escherichia v gapA (3)</i>   | —  | —  | —  | —  | ?  |
|                                |    |    |    |    |    | <i>Escherichia f gapA (3)</i>   | —  | —  | —  | —  | ?  |
| <i>Ustilago</i>                | —  | —  | —  | —  | —  | <i>Salmonella t gapA</i>        | —  | —  | —  | —  | ?  |
| <i>Agaricus GPD1,2</i>         | —  | —  | —  | —  | —  | <i>Salmonella sp. gapA (15)</i> | —  | —  | —  | —  | ?  |
| <i>Phanerochaete</i>           | —  | —  | —  | —  | —  | <i>Citrobacter gapA</i>         | —  | —  | —  | —  | ?  |
| <i>Schizophyllum</i>           | —  | —  | —  | —  | —  | <i>Enterobacter gapA</i>        | —  | —  | —  | —  | ?  |
| <i>Podospira</i>               | —  | —  | —  | —  | —  | <i>Klebsiella gapA</i>          | —  | —  | —  | —  | ?  |
| <i>Claviceps</i>               | —  | —  | —  | —  | —  | <i>Serratia m gapA</i>          | —  | —  | —  | —  | ?  |
| <i>Cryphonectria</i>           | —  | —  | —  | —  | —  | <i>Serratia o gapA</i>          | —  | —  | —  | —  | ?  |
| <i>Glomerella</i>              | —  | —  | —  | —  | —  | <i>Escherichia c gapB</i>       | —  | —  | —  | —  | —  |
| <i>Curvularia</i>              | —  | —  | —  | —  | —  | <i>Escherichia c gapC</i>       | —  | —  | —  | —  | ?  |
| <i>Cochliobolus</i>            | —  | —  | —  | —  | —  | <i>Anabaena gap1</i>            | —  | —  | —  | —  | —  |
| <i>Aspergillus</i>             | —  | —  | —  | —  | —  | <i>Anabaena gap2</i>            | —  | —  | —  | —  | —  |
| <i>Kluyveromyces</i>           | —  | —  | —  | —  | —  | <i>Anabaena gap3</i>            | —  | —  | —  | —  | —  |
| <i>Zygosaccharomyces</i>       | —  | —  | —  | —  | —  |                                 |    |    |    |    |    |
| <i>Saccharomyces Gap1,2,3</i>  | —  | —  | —  | —  | —  | <i>Chondrus GapA/B</i>          | —  | —  | —  | —  | —  |
|                                |    |    |    |    |    | <i>Gracilaria GapA/B</i>        | —  | —  | —  | —  | —  |
| <i>Phytophthora</i>            | —  | —  | —  | —  | —  | <i>Chlamydomonas GapA/B</i>     | +  | —  | +  | —  | —  |
| <i>Dictyostelium</i>           | —  | —  | —  | —  | ?  | <i>Zea GapA</i>                 | —  | —  | —  | +  | —  |
| <i>Entamoeba</i>               | —  | —  | —  | —  | ?  | <i>Arabidopsis GapA</i>         | —  | —  | —  | +  | —  |
| <i>Trypanosoma b gapG</i>      | —  | —  | —  | —  | —  | <i>Pisum GapA</i>               | —  | —  | —  | +  | —  |
| <i>Trypanosoma c gapG</i>      | —  | —  | —  | —  | —  | <i>Arabidopsis GapB</i>         | —  | +  | —  | +  | +  |
| <i>Leishmania gapG</i>         | —  | —  | —  | —  | —  | <i>Pisum GapB</i>               | —  | +  | —  | +  | +  |
| <i>Trypanosoma b gapC</i>      | —  | —  | —  | —  | —  |                                 |    |    |    |    |    |
| <i>Leishmania gapC</i>         | —  | —  | —  | —  | —  |                                 |    |    |    |    |    |
| <i>Trichomonas</i>             | —  | —  | —  | —  | —  |                                 |    |    |    |    |    |
| <i>Giardia</i>                 | —  | —  | —  | —  | ?  |                                 |    |    |    |    |    |

Distribution of the five shared introns among the 116 GAPDH genes of known exon/intron structure. Plus sign, presence of an intron; minus, absence of intron; ?, missing data. Intron positions are numbered according to ref. 1. Genes are organized from top to bottom by major phylogenetic groups: plants/algae, animals, fungi, protists, eubacteria and nuclear genes of plastid/eubacterial ancestry. All sequences are available from GenBank except for *Dictyostelium* (from M. Smith and R. Doolittle) and *Gracilaria* (from Y.-H. Zhou and M. Ragan). Multiple GAPDH sequences from the same eukaryotic species are grouped on the same line if they have the same genomic structure, except for the anciently diverged *gapC* (cytoplasmic) and *gapG* (glycosomal) of trypanosomatids. Multiple GAPDH genes from the same eubacterial species are shown on separate lines because they represent ancient duplications. Numbers in parentheses indicate numbers of isolates within a eubacterial species whose orthologous (*gapA*) genes have been sequenced. Multiple species of the same genus are distinguished by the first letter of the species name. The *Chlamydomonas*, *Chondrus* and *Gracilaria* nuclear-encoded plastid GAPDH genes are denoted *GapA/B* because their relationships to land plant *GapA* and *GapB* genes are unclear.

SIR — “Lightning never strikes the same place twice”, declares a common, but incorrect, aphorism. If lightning struck with equal probability at every possible location, multiple events would indeed be vanishingly rare. However, chances vary from site to site, so that some sites are struck repeatedly, and others not at all.

Kersanach *et al.*<sup>1</sup> have found five matching intron positions between the ten positions known among genes of the *gapAB* family (eukaryotic nuclear genes





Probability that at least 5 of 10 introns randomly inserted in one gene will match any of the 42 intron positions in another gene, as a function of target sites available for insertion (data from computer simulations).

of chloroplast origin) and the forty-two positions for *gapC* genes. Separate insertions cannot explain this pattern, they argue, and these spliceosomal introns must therefore have been inherited from an ancestral gene. The authors suggest that the introns predate the divergence of eubacteria and eukaryotes, even though spliceosomal introns have been found only in genes in the eukaryotic nucleus<sup>4</sup>, and not in the thousands of genes examined in prokaryotes.

Kersanach *et al.*<sup>1</sup> examine the possibility of separate insertions using a model in which introns insert "randomly with a homogeneous probability"<sup>7</sup> at each possible site. The resulting probability of five matching positions,  $2.2 \times 10^{-5}$ , allows them to exclude this model. But a homogeneous insertion probability would not have been expected, since mobile elements typically exhibit heterogeneous probabilities, striking some sites more often than others. Many examples of this principle could be given, the most relevant being the recent demonstrations<sup>8,9</sup> of the mobility of group II self-splicing introns (distant relatives, perhaps, of spliceosomal introns), which included evidence of recurrent insertions into the same site<sup>8</sup>, and sequence similarities among different insertion sites<sup>9</sup>.

The greater the preferences, the greater the chance that introns inserted into separate copies of a gene will match. The figure shows this effect for an idealized pair of *gap* genes. The chance of obtaining five or more matches goes up dramatically as the number of target sites for insertion goes down. For instance, if introns insert at a 2-nucleotide target site, only 62 (on average) of the 998 possible intron positions (in a 333-codon *gap* gene) would be targets, and five (or more) matching intron positions would not be rare, but quite common ( $P > 0.9$ ). This idealized treatment ignores nucleotide sequence divergence, which must have slowly reduced the

tendency for preferred insertion sites (and thus, intron insertions) to coincide. Nevertheless, this is a comparatively minor effect, as the sequences of *gapAB* and *gapC* genes are still quite similar.

Either spliceosomal introns are mobile elements that can insert at matching positions in different copies of a gene, or they are ancient relics lost from nearly all bacterial genes. The difficulty with the latter position lies in explaining why the tiny fraction of bacterial-derived genes that have 'retained' introns (for example *gapAB* genes) is so conspicuously identical with the tiny fraction of bacterial-derived genes that (due to lateral transfers) resides in the nucleus (for example *gapAB* genes). Compared to this riddle, explaining a few matching intron positions is simple: spliceosomal introns, like lightning and, more importantly, like other mobile elements, often strike the same place twice.

#### Arlin Stoltzfus

Department of Biochemistry,  
Dalhousie University,  
Halifax, Nova Scotia B3H 4H7,  
Canada

CERFF *ET AL.* REPLY — Logsdon/Palmer and Stoltzfus suggest that the five identical intron positions across chloroplast and cytosolic GAPDH genes (*GapA/B* and *GapC*, respectively) are best explained by parallel insertions at common target sites rather than by common ancestry. But if relatively late targeted intron insertions played a major role in GAPDH gene evolution, one would expect to find coincident intron positions preferably across the related *GapC* genes of plants, animals and fungi, rather than across the highly divergent paralogous gene types *GapA/B* and *GapC*. Precisely the opposite is observed. Only one GAPDH intron (No. 15 in Fig. 1 of ref. 1) fulfils the former criterion, whereas five fulfil the latter. Phrased another way, why does lightning (Stoltzfus), if it strikes twice, strike preferentially at distantly related 'protosplice sites' in the most divergent genes and avoid more closely related ones in genes of more recent common descent? Without invoking complex auxiliary assumptions, the 'introns-late' hypothesis cannot account for this observation in GAPDH genes, while retention of ancient introns and differential intron loss ('introns-early' hypothesis) readily explain the data.

Logsdon and Palmer's rationale in favour of a late lineage-specific acquisition of GAPDH introns is based to a large extent on negative evidence: lack of introns in many GAPDH genes. For example, *Arabidopsis GapC* lacks three introns (Nos 34, 44 and 46 in Fig. 1 of ref. 1) which are present in maize and pea *GapC*: three losses in *Arabidopsis* are more likely than six independent gains at three identical positions in pea and maize. There are

other such examples which clearly contradict the *a priori* notion of Logsdon and Palmer that intron gain should be generally more plausible than intron loss. Ironically, Logsdon and Palmer, who support intron mobility<sup>4</sup>, also reject categorically the idea of 'intron slippage'<sup>10,11</sup>, the occasional displacement of an intron over a short distance of a conserved coding sequence (not to be confused with 'intron sliding', the sliding of a single intron junction leading to insertions/deletions<sup>12</sup>) and on the basis of this implicit axiom "once inserted, always immobile" invoke "too tiny" ancestral GAPDH exons as evidence against the exon theory of genes. We maintain that the numerous cases of quasi conservation or clustering of GAPDH introns may be best explained by intron slippage and not by independent intron insertion, which is particularly evident for introns 37 and 38 which are separated by one nucleotide in *GapC* genes of three related basidiomycetes sharing several additional introns at identical positions (see Fig. 1 of ref. 1). We have previously argued<sup>10,11</sup> that both loss and slippage of introns can be rationalized in terms of rare, occasional splicing errors leading to modified pre-mRNAs which can then be re-introduced into the genome via reverse transcription and gene conversion.

Finally, 'introns late' supporters need to address the critical question at hand, how were genes and long contiguous open reading frames assembled in early evolution? If they do not accept the concept of intron-mediated 'exon-shuffling' for primordial gene evolution<sup>6</sup>, then they should suggest some plausible alternative hypothesis with testable predictions. In conclusion, the GAPDH gene system is the only current example of ancient gene diversity comprising a sufficiently large dataset to allow direct discrimination at the gene level between the 'introns-early' and the 'introns-late' view and it clearly provides positive evidence in favour of the former. The recent discovery of a 'classic' GAPDH in the archaeobacterium *Haloarcula vallismortis*<sup>13</sup> and the characterization of the corresponding operon (unpublished data) indicate that the *GapA(B)/GapC* gene duplication, indeed, occurred

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in the progenote. As information from other genes of similar antiquity becomes available, more clarity should ensue. It is the data, rather than arguments, that are lacking.

**Rüdiger Cerff, William Martin**

**Henner Brinkmann**

*Institut für Genetik,*

*Technische Universität Braunschweig,*

*D-38023 Braunschweig, Germany*

## Can female adders multiply?

**SIR** — Madsen *et al.*<sup>1</sup> conclude from a field study on an adder (*Vipera berus*) population from southern Sweden that most of the females mated multiple times in a season, and that those females that copulate more frequently than others also produce a higher mean number of viable offspring. These findings suggested to the authors that the increase in viability of fertilized eggs is due to sperm competition, in which the 'best' sperm compete for the chance to effect fertilization. But in an adder population from northeastern Italy (Sella Nevea, Carnic Alps, 1,100 m high), we find that only about 18% of the females mated multiple times in a season, and those females copulating multiple times very often do so with the same male<sup>2</sup>.

In the reproductive period of 1993, we captured 20 free-living adders (belonging to the population studied by Luiselli<sup>2</sup>) immediately after the end of hibernation (before the start of the mating period), and placed them in a outdoor enclosure (in the adder habitat) to monitor the exact number of copulations of each female. Receptive males from the same population were introduced into the enclosure. Ten female individuals were mated only once (group A), and ten were mated multiple times (from 3 to 8, group B). We used differently sized males, but there were no mean size differences between adders that copulated with female groups A or B. After the end of the experiments, we measured clutch parameters of the two groups of females by using methods as described in refs 3 and 4.

As in the study by Madsen *et al.*<sup>1</sup>, we found that the number of copulations was not significantly correlated with litter size or with female fecundity relative to body size (in either case,  $r < 0.3$ ,  $P > 0.1$ ), and that the number of matings by a female did not affect her mean offspring mass, her total mass or her proportional body mass loss during gestation (in all cases,  $r < 0.3$ ,  $P > 0.1$ ). But our data did differ from those of Madsen *et al.*<sup>1</sup> in that multiple matings did not reduce the proportions of offspring that were dead at birth. In fact, the proportion of dead offspring per litter was not significantly different in the two groups of females ( $\bar{x} = 12.0 \pm [s.d.]$

16.52% of female group B versus  $14.0 \pm 17.66\%$  of female group A: two-tailed  $t = -0.19$ , d.f. = 18,  $P > 0.8$ ), and the correlation between proportion of still-born young and number of different males mated with was not significant ( $r = 0.53$ , ANOVA: mean square = 0.069,  $F = 3.17$ ,  $P = 0.1$ ). Thus, at least in the population we studied, the number of different males mated with does not seem to be the primary determinant of the proportion of viable offspring produced by a female adder. Thus, although the arguments of Madsen *et al.*<sup>1</sup> are interesting, we suspect their results may not apply to all adder populations. On the other hand, it could be claimed that the mating pattern is adaptive in each case, and that female adders mate multiply with different males only when there is a positive benefit from doing so.

**Massimo Capula**

**Luca Luiselli**

*Department of Animal and Human Biology,*

*University of Rome "La Sapienza",*

*via Alfonso Borelli 50,*

*I-00161 Rome, Italy*

**SIR** — Females of many animal species mate frequently, with several different males. This 'promiscuous' female behaviour is unexpected from simple Darwinian theory because the number of offspring produced by a female does not increase if she has more sexual partners. In an earlier paper<sup>1</sup>, some of us suggested that by this behaviour, females promote sperm competition among males, and their offspring are thereby sired by males with 'better' genes. We found that female adders (*Vipera berus*) that mated with several males produced a higher proportion of viable offspring than did 'monogamous' females<sup>1</sup>. Parker<sup>5</sup> suggested that judgement of this hypothesis should be suspended until further evidence was accumulated. Here we report a study of lizards that strongly supports the earlier hypothesis<sup>1</sup>. Not only does multiple mating of lizards with different partners increase hatching success and lower the incidence of deformities, but it also enhances survivorship of free-living juveniles.

We studied a population of marked, blood-sampled sand lizards (*Lacerta agilis*) 50 km south of Gothenburg on the Swedish west coast<sup>6,7</sup>. Matings were directly observed ( $n=32$ ) or were inferred from the post-copulatory mate guarding ( $n=108$ ) that characteristically follows the 2–4-min-long copulation<sup>7</sup>. In 1989 and 1990, we incubated eggs from the female lizards under identical conditions in the laboratory. Hatchlings were marked by toe-clipping and were blood sampled before being released at random sites at the Asketunna study area. We used DNA fingerprinting<sup>7</sup> to establish paternity and to assess the degree of genetic variation in

the population. After one year we recaptured the survivors to determine whether offspring from multiply-mating females were more likely to survive as free-living juveniles.

Genetic variation in the population was low (mean band sharing among individuals was 66%, range, 63–68,  $n=30$ ). Despite the low genetic variation, we could identify male-specific bands in five broods; four had mixed paternity (our unpublished data). Females mated on average 3.7 times with 1–5 different males (mean, 1.7). The resulting clutches varied in hatching success (mean, 81%; range, 38–100). We recaptured 42 of the 516 released hatchlings, with some clutches being much more highly represented than others (mean, 9.5%; range, 0–43%). As some of us predicted (one-tailed tests), a female's number of sexual partners was: (1) positively correlated with the hatching success of her eggs ( $r_s=0.59$ ,  $P=0.0003$ ,  $n=31$ ); (2) negatively correlated with the proportion of hatched young that exhibited malformations ( $r_s=0.33$ ,  $P=0.035$ ,  $n=31$ ); and (3) positively correlated with the proportion of her offspring that were recaptured after 1 year ( $r_s=0.37$ ,  $P=0.020$ ,  $n=31$ ). This result remained significant when clutch size was controlled for in a partial correlation analysis ( $r_s=0.41$ ,  $P=0.014$ ,  $n=31$ ).

Could these differences in offspring viability be caused by nutrients in the ejaculate, rather than by genetic enhancement of offspring? Probably not. Some females mated more than once with the same male, enabling us to examine the effects of number of copulations independently of the number of partners. Increased copulations with the same male did not increase a female's offspring viability (number of matings versus hatching success:  $r_s=-0.19$ ,  $P=0.502$ ; versus % deformities:  $r_s=0.42$ ,  $P=0.136$ ; versus offspring survival:  $r_s=0.41$ ,  $P=0.143$ ,  $n=15$ ).

**Mats Olsson\***

*Department of Zoology,*

*University of Göteborg,*

*S-413 90 Göteborg, Sweden*

**Annica Gullberg**

**Håkan Tegelström**

*Department of Genetics,*

*Uppsala University,*

*S-750 07 Uppsala, Sweden*

**Thomas Madsen**

**Richard Shine**

*School of Biological Sciences,*

*The University of Sydney,*

*NSW 2006, Australia*

\*Present address as for T.M. and R.S.

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# The essence of emergence

Michael Berry

**The Quark and the Jaguar: Adventures in the Simple and the Complex.** By Murray Gell-Mann. W. H. Freeman/Little, Brown: 1994. Pp. 392. \$23.95, £18.99.

WHILE walking in a South American rainforest and thinking about quantum mechanics, Murray Gell-Mann encountered a jungle cat. They stared at each other for a while, then the cat turned away and disappeared among the trees. Gell-Mann employs this meeting as a metaphor for the central question to which his book is devoted: how can the fundamental laws of physics, pre-eminent of which is quantum mechanics, conducting an orchestra of elementary particles such as quarks, produce a diversity of complex organisms such as the jaguar? This is, on the grandest scale, the problem of reduction, that is, of intellectual continuity between the partial descriptions provided by different sciences on different levels. He is brave to attempt this, because of the prejudice "that serious work is restricted to beating to death a well-defined problem in a narrow discipline, while broadly integrative thinking is relegated to cocktail parties". The attempt is heavily influenced by his recent experiences at the Santa Fe Institute for the study of complex systems, an institute he helped to set up.

An immediate difficulty, at the lowest level of the chain of connections, is with the interpretation of quantum mechanics. A jaguar is a unique, persisting and well-defined classical object, whereas an elementary particle is a quantum object, identical to its companions, transitory and with properties that depend on how it is observed. Gell-Mann's view, extensively developed by many people over several decades and admirably described here in a nontechnical way, is that a classical history emerges by summing over all the quantum possibilities compatible with it. This is 'coarse-graining'; and the sum, over the 'fine-grained' alternatives, induces 'decoherence' that eliminates the interference phenomena (for example, between the live and dead Schrödinger cat) at the root of all characteristically quantum effects. On this view, with which I agree but which is controversial (although Gell-Mann does not emphasize the fact), there is no need to supplement quantum mechanics by, for example, adding nonlinear terms to the Schrödinger equation. As a treatment of the classical limit it is, however, incomplete, because it rules out of consideration the subtle and intricate emergent phenomena, now being discovered, that occur in systems whose environment is so well controlled that decoherence does not happen.

A classical world is of course far from a

living one; indeed, the alleged sterility of the deterministic world of Newtonian bouncing balls is often contrasted with the changing richness of the natural world. A central concept in understanding the latter is the 'complex adaptive system', which "acquires information from its environment . . . identifying regularities in that information, condensing these regularities into . . . a 'schema', and finally acting in the 'real world' on the basis of that schema". Gell-Mann gives a careful discussion of the various sorts of complexity, starting with chaos (that is, unpredictability arising from dynamical instability, so that initially neighbouring trajectories develop very differently). Closely related is the algorithmic complexity of a sequence of symbols, defined as the length of the shortest computer program that could generate it. These ideas are intended to describe the absence of order, and fail to capture the intimate blend of structure and randomness in a complex adaptive system. For this, a better concept is effective complexity: "the length of a concise description of the regularities of [a] system". There is much discussion of how the high effective complexity of a complex adaptive system could arise as an emergent phenomenon, from simple rules

acting over a long time.

In the final chapters, there is a sudden shift away from science, with a discussion, in a voice "as much that of the advocate as of the scholar" of the principles that should govern transitions to a world where human activity preserves and sustains a diversity of species and cultures. Although I have some sympathy with Gell-Mann's opinions, I find his arguments less compelling than the rest of the book. For a start, there is the fundamental question of why it is good to preserve diversity. He asks: "Does it make any sense to destroy . . . [what] evolution has built up over such a long period?" But (as he mentions) this has happened before, for example at the end of the Cretaceous period, and the eventual result was more diversity, including us. By inhibiting our 'natural' destructiveness, could we not be committing the ultimate 'speciesist' crime of preventing the emergence of organisms more effectively complex and adapted — in a word, better — than us? And is it desirable to preserve cultures with values (such as intolerance and expansionism) incompatible with our own? Gell-Mann recognizes this difficulty but has nothing new to say about it.

As well as the thoughtful exploration of its main themes, the book contains many insights into superstition, pseudoscience ("the dissociation of belief from evidence") and scientific creativity, and a great deal of wry and engaging humour. □

Michael Berry is in the H. H. Wills Physics Laboratory, University of Bristol, Tyndall Avenue, Bristol BS8 1TL, UK.



This decorative cloth, from the third century BC, was found in the desert near Pisco, 125 miles south of Lima. The picture is taken from *The Land of the Incas*, first published in 1977. The book contains more than a hundred spectacularly beautiful photographs by Hans Silvester of the landscape, archaeology and people of the region, together with a short introduction to the Inca empire by Jacques Soustelle. Thames and Hudson, £12.95 (pbk).

## Science at bay

Bob Johnstone

**Science Has No National Borders: Harry C. Kelly and the Reconstruction of Science in Postwar Japan.** By Hideo Yoshikawa and Joanne Kauffman. MIT Press: 1994. Pp. 133. \$22.50, £19.95.

In November 1945, engineers from the US Sixth and Eighth Armies dismantled the two cyclotrons at Japan's elite national laboratory, the Physical and Chemical Research Institute (Riken), and dumped them in Tokyo Bay. Other cyclotrons at Kyoto and Osaka Universities were treated similarly.

The Occupation Forces justified the destruction on the grounds that the cyclo-

duced a sense of dread. Which laboratory would be the next to fall victim to the whims of the Occupation Forces?

Scientists at Hokkaido University's Microwave Research Institute were particularly apprehensive. They had built an experimental 'death ray', equipment designed to melt aircraft metal using high-energy electromagnetic waves. Fearing that the apparatus would be destroyed, and that they would be charged with war crimes, the scientists hid their equipment in a schoolhouse in a nearby village.

Much to their surprise, the representative sent from General Headquarters, Tokyo, to investigate their work did not seem interested in reprisals. He was Harry C. Kelly, a 37-year-old American physicist who had been appointed deputy chief of the Scientific and Technical Division of the Economic and Scientific Section, a civilian branch of the Occupation Forces. It was one of his first assignments in Japan.

Having persuaded the Japanese scientists that it would be better for them to come clean and show him their gear, Kelly quickly decided that the prototype death ray, with a range of 20 feet at best, would never be of much military use. But the equipment could be useful for other, peaceful purposes, so, to forestall any further destruction by military personnel, Kelly hung a large sign over the apparatus, which read: "Property of Hokkaido University, Electronic Laboratory, inspected by H. C. Kelly, General Headquarters".

This enlightened and decisive action was characteristic of Kelly, as Yoshikawa and Kauffman make clear:

Industrious, ambitious, and thoughtful, Kelly traveled throughout Japan, going well beyond the limited scope of the intelligence function he had been sent over to pursue. He believed that establishing an enduring democracy in Japan depended at least in part in the country's economic rehabilitation, and that science would necessarily play a key role in bringing about that rehabilitation. At the same time, he found Japan's scientific establishment deeply entrenched in research traditions that overemphasized basic science, with scant attention to the kind of applied research and development that the country needed to meet its immediate economic needs. Many of the young Japanese scientists Kelly met shared his assessment and his desire to reorganize Japan's scientific establishment.

By introducing a policy of mutual trust and by using his resourcefulness to break through bureaucratic barriers, Kelly helped save many scientists and laboratories from punitive action in the early years of the occupation, facilitated the reentry of the Japanese scientific community into the international arena, and helped reorganize Japan's scientific and technological base.

The Japanese were duly grateful to Kelly for his efforts. They awarded him the first honorary membership granted by

the Physical Society of Japan and, in 1969, the Order of the Sacred Treasure, Second Class — the highest honour the Japanese government bestows on foreigners. Now, as a further tribute, some 18 years after Kelly's death, comes this excellent book. If there was ever a labour of love, this is it.

The book is the English-language edition of a work that originally appeared in Japanese. But it is no mere translation. Kauffman, who is in the Department of Political Science at the Massachusetts Institute of Technology, has done a marvellous job of restructuring the book for a non-Japanese readership, adding a considerable amount of necessary background information. This is especially welcome given the scant literature on this fascinating period (and indeed, on the history of twentieth-century Japanese science and technology in general).

Although a slim volume, it is densely packed and includes much incidental information of interest to students of the period. For example, it is often forgotten that while Americans predominated, the occupation also included representatives of the other Allied powers. Kelly's boss in the Scientific and Technical Division was an Australian, Brigadier John O'Brien. He and another Australian, Lieutenant Colonel Ted Allen, were responsible for important changes in the Japanese government's style of doing research. Most notable was the creation of the Agency for Industrial Science and Technology, the arm of the Ministry of International Trade and Industry that runs the ministry's (in)famous national research projects.

Kelly was instrumental in setting up key elements of the relationship between science and government in early postwar Japan. These included the Science Council, Japan's unique 'parliament of scientists', and the now defunct Scientific and Technical Administration Commission. Unfortunately, both organs failed to live up to their creators' ideals, as a result of politicization and competition with other, more powerful bureaucracies.

In 1950, after four hectic years, Kelly left Japan to take up a position as assistant director for scientific personnel and education at the newly formed National Science Foundation in Washington, DC. But he always kept his ties with Japan. In 1961 he became the first American co-chairman of the Japan-United States Committee on Scientific Cooperation, a body whose very existence indicates the extent to which Japanese science had recovered its vitality. And after Kelly's death, some of his ashes were brought to Japan for burial next to the grave of his friend Yoshio Nishina, the physicist whose cyclotrons had been dumped so unceremoniously in Tokyo Bay. □

Bob Johnstone is Japan correspondent for *Wired*.



Caricature of Kelly by H. Tamiya, 1947.

trons had been used during the Second World War to produce uranium isotopes, as part of Japanese efforts to develop an atomic weapon. But Japan never came close to this goal and by late 1945 Japanese scientists were keen to resume research that had been cut short by the war. For this they needed radioactive isotopes, and cyclotrons were then the most effective means of producing them.

The destruction of the cyclotrons was denounced by scientists elsewhere, most notably those at Oak Ridge and Los Alamos in the United States, who likened it to the burning of books. Within the Japanese research community, it pro-



# Better late than never

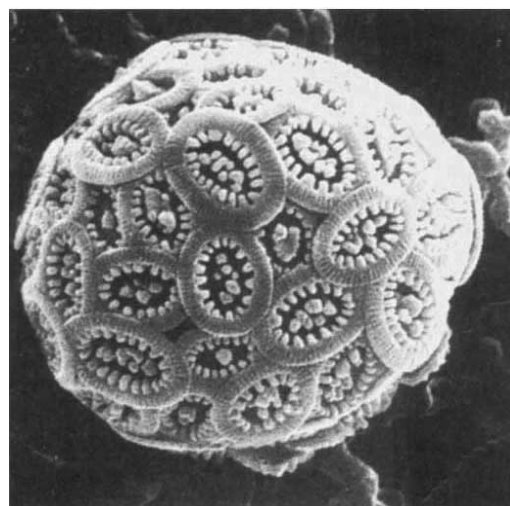
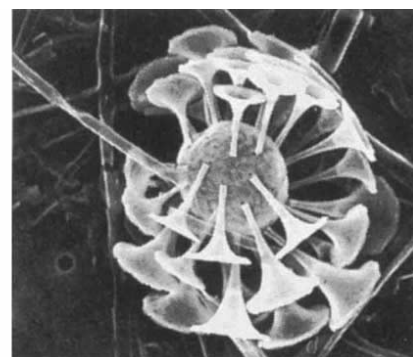
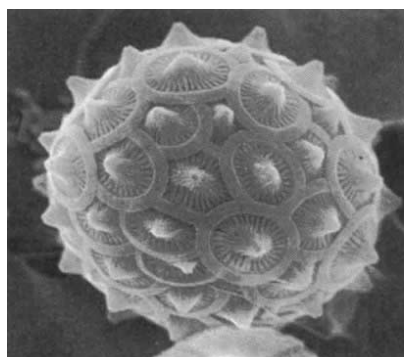
Paul Bahn

**Timewalkers: The Prehistory of Global Colonization.** By Clive Gamble. *Harvard University Press/Alan Sutton: 1993. Pp. 309. \$27.95, £19.99.*

THE award for the silliest archaeological book title of 1993 must go to *Timewalkers*. What on Earth does this word mean? The author never actually defines it, but seems to use it in place of 'ancient humans'. It reminds one of those snappy single-word titles for television series. 'Timewalkers' is not simply a dreadful neologism that sounds like a sci-fi term (a hybrid of Timelords and Luke Skywalker, perhaps?), it is also utterly devoid of meaning: as walking is inherently and inevitably a diachronic action, then presumably I shall become a Timewalker when I pop out to take this review to the Post Office — as soon as I have finished being a Timetyper. Since the theme of this book is the prehistoric colonization of the globe, one would have thought that 'Colonizers' or even 'Landgrabbers' would have been a more apt choice.

The author's aim is to present a new kind of world prehistory which, laudably, places the emphasis not so much on stone tools or skeletal shapes — although these inevitably dominate the text at times — but rather on behaviour and social context. After an introductory section in which Gamble presents the familiar evidence for early explorers and Victorian scholars being patronizing racists with regard to the "primitive" peoples in remote parts of the globe, he leads us through the whole of prehistory, at an accelerating pace: lots of space is devoted to the earliest hominids for which we have the least evidence, but by the end of the last ice age we are at full gallop, with Holocene colonizations of empty islands accorded only a few pages.

At first glance the book is thorough and reasonably well-balanced. But it quickly becomes apparent that the author has his own agenda, and his treatment of the evidence is clearly tempered by the particular version of world prehistory that he happens to favour. In particular, he sees a definite break at around 50,000 years ago between what he calls the "Ancients" and ourselves, the "Moderns"; in essence this boils down to stating that the Neanderthals were so different from ourselves that a firm line can be drawn between them and us, a view that is by no means universally held. To shore up this approach, all the growing body of evidence for 'art' before 40,000 years ago is simply dismissed and ignored.



**COCCOLITHOPHORES**, one of the oceans' primary algal groups, are important in palaeo-environmental reconstruction and stratigraphy. These scanning electron micrographs ( $\sim \times 6,000$ ) appear in *Coccolithophores* edited by Amos Winter and William G. Siesser, which covers the history of work on the species, their biology and geochemical aspects, and the form, function and classification of their skeletal elements. The centrepiece is a comprehensive atlas chapter, with 140 micrographs. Cambridge University Press, £75, \$150.

In fact, throughout the book, later dates are consistently preferred to earlier ones: for example, the author selects the youngest date available for the Israeli site of 'Ubeidiyah (although new work has recently brought further support for a far earlier date); he is clearly uncomfortable with the (apparently well-founded) date of 1.6 million years ago for the jaw from Dmanisi, Georgia; and Yuri Mochanov's claims for a very early occupation at Diring, Siberia, are also given short shrift, although they are beginning to find some support in the United States where specialists agree that the lithics are artefacts and thermoluminescence dates of 500,000 years have been obtained from soil samples from the site. Gamble also simply dismisses, without discussion, all the claims for people in Europe earlier than Isernia la Pineta, Italy, at 730,000 years ago — so much for Vallonnet in France, or Chilhac, let alone the growing number of claims for even earlier occupation from Spain to Romania. He opts for the later dates for the Palaeolithic of Japan; in Australia he appears to accept the thermoluminescence evidence for humans at 55,000 or 60,000 years ago, but omits not only Gurdip Singh's (albeit contested) pollen and charcoal evidence for a human presence at 120,000, but also Peter Kershaw's recent findings of similar pollen and charcoal evidence at 140,000 years ago on the continental shelf off northeast

Queensland, the only such disturbance in a 1.5-million-year record. Worst of all, he makes the astounding claim that only two sites in the whole of the New World (Meadowcroft and Monte Verde) survive as evidence of a possible early settlement, thus ignoring completely not only Pedra Furada — potentially the most important archaeological site in the Western Hemisphere — but also many others.

One cannot object to claims for early dates and sites being subjected to critical or even harsh scrutiny; but the author can make no excuse for simply ignoring or brushing aside so much material that conflicts with his chosen scenario. By contrast, he accepts other claims quite ingenuously — for instance, that the Laetoli trail in Tanzania has a child treading in an adult's footsteps — and his reasoning at times seems inconsistent. He supports the view that the wealth of art and ornament in the Upper Palaeolithic of central and eastern Europe represents a "hysteria" in reaction to a hard life where economic and social survival was a considerable problem; yet he fails to address the question of what could explain the similar explosion in southwest Europe, an area where we have no evidence for any such struggles, and indeed where the living seems to have been easy, as far as we can tell.

Unfortunately, we cannot tell very far. The fundamental problem with trying to

put the emphasis on behavioural and social aspects of the past, and especially the very remote past, is — as the author is well aware — that one is often reduced to pure speculation: one need only refer to the endless, infinitely ingenious theories of why hominids became bipedal. This does not mean that it is not worth trying to take a fresh look at the evidence — indeed Gamble is to be admired for having a go at such an intractable problem — but alas it does not seem to lead to any new insights. The book's blurb claims that it "throws a striking new light into the background of human history", but in fact the concluding chapter merely decides that people colonized the world purposefully rather than through aimless drifting or random diffusion. Their only direction or goal was to survive and reproduce their existing social life, and the motor of prehistory was behaviour ungoverned by natural laws but constrained by the practicalities of existence.

In other words, people rather than penguins ended up all over the globe because they were people with rational minds who could construct and change their worlds. But this is pretty similar to

the traditional view that people went to the ends of the Earth 'because they were there' or because there was nowhere else to go. It seems to come down to 'people colonized the Earth precisely because they were people'. Nevertheless, in presenting the theories of how they became people in the first place, and in attempting to chart how and when and even why the various stages of global colonization occurred, Gamble has produced a readable and novel (I use the word advisedly) account of prehistory.

I am not sure whom the book is aimed at, however. It is certainly not a textbook, and although it presents a (highly selective and skewed) establishment view of human evolution, readers will find several more user-friendly and richly illustrated volumes on the market. Gamble's text is often dense, and his love of buzz-words such as 'exaptation' or 'teleology', and his use of obscure terms such as 'vicariance biogeography', are likely to alienate students and public alike. □

*Paul Bahn is a freelance writer on archaeology at 428 Anlaby Road, Hull HU3 6QP, UK.*

roots of computer art still seem to undermine its artistry.

The book includes some strange excursions. Charles Perry, the sculptor responsible for the beautiful aluminium "Eclipse" in a San Francisco hotel and the bronze "Continuum" in the National Air and Space Museum, Washington, DC, writes an honest and self-effacing essay, inappropriately entitled "The Role of the Artist's Intuition in Science". He describes himself as one of the many dreamers gathered at the frayed edges of science, fraught with wishful intuitions about the Universe, and gives a cheerful tour of the background to his geometrically complex sculptures. Harriet Brisson, in "Visualisation in Art and Science", is less modest: "Visual imagery may serve as a bridge between the human spirit and the complicated technical language of the twentieth century".

The overall impression of the book is of mathematicians enjoying exploring and creating beautiful objects and spaces (without the help of artists, thank you) and a few artists joining in (with varying degrees of humility). The essentially commercial work of developing tools for a wide range of computer users continues alongside these recreations. "The 1990s will be the decade of visual processing", announces Silicon Graphics, manufacturers of visualization tools and workstations. Whether people value artists' contribution will be tested in the time-honoured way: by asking them to pay for it. □

*Nick Beard is director of information services at Health Care International, Beardmore Street, Clydebank G81 4DY, UK.*

## Picturing mathematical information

*Nick Beard*

**The Visual Mind: Art and Mathematics.** Edited by Michele Emmer. MIT Press: 1993. Pp. 274. \$39.95, £35.95.

*The Visual Mind* is about the overlap between art and mathematics, and it is replete with examples, many from the days before computer graphics. It is written by mathematicians and artists, some of whom claim to be both. Although the blend of art and mathematics will not seem odd to mathematicians (and not just topologists, nonlinear-systems analysts and computer scientists), the book's thesis is that the interplay is mutually beneficial. Evidence of mathematics' continuing contribution to art is not in dispute. But evidence of a reciprocal contribution is sparse indeed.

There are choices to be made in building representations, especially visual ones. The mapping of abstract mathematical data to real images is arbitrary, yet important decisions, many of them aesthetic, must be made in selecting one map over another. Perhaps artists can play a valuable part? But graphics are at once easier and harder to understand than raw data. Their appeal is in the swiftness with which one can grasp the intended message. The difficulty is the ease with which graphically represented data can be misread, masked or misinterpreted. Unfortunately, for all their insight and intuition,

many artists fail to appreciate these subtleties: recall the flurry of innumerate prose penned on chaos and fractals.

There is more to this book than fractals, however. There are many examples of weird and wonderful mathematical forms, most of which, before computer graphics, were effectively invisible except in the imaginations of mathematicians. Tiling, tessellation, topology, trigonometry and technology all loom large. There is much here to interest a wide range of readers. *The Visual Mind* is an attractive and enjoyable work, despite its uneven demands on readers' mathematical abilities.

The opening essay is Max Bill's "The Mathematical Way of Thinking in the Visual Art of Our Time", first published in 1949. It is intriguing that the first line should now appear so dated: "By a mathematical approach to art it is hardly necessary to say I do not mean any fanciful ideas for turning out art by some ingenious system . . . with the aid of mathematical formulas." Art *can* be algorithmic: William Lathan and Harold Cohen, for example, have written software to produce art. However, digitally produced images have no 'original'. The art critic Peter Fuller writes of the "bogus religiosity" attached to "original works", even when most observers cannot tell an original from a copy. Yet for some people, the lack of an original and the apparently formulaic

### New in paperback

**The Structure and Confirmation of Evolutionary Theory** by Elisabeth A. Lloyd. Philosophy of Darwinism, first published in 1988. Princeton University Press, \$14.95, \$11.95.

**The Uses of Life** by Robert Bud. A well-received history of biotechnology. Cambridge University Press, £12.95, \$19.95.

**Exploding the Gene Myth** by Ruth Hubbard and Elijah Wald. "A broad overview of the science of genetics and its social implications" (Dorothy Nelkin, *Nature* 363, 27; 1993). Beacon Press, \$12.

**Land Degradation** by C. J. Barrow. An introduction to this "key issue in world conservation". Cambridge University Press, £19.95, \$29.95.

**The Royal Society and Its Fellows, 1660–1700: The Morphology of an Early Scientific Institution** by Michael Hunter. Revised edition of this standard source, first issued in 1982. British Society for the History of Science, £10 (£8 for members).



# Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand

Frederic J. de Sauvage, Philip E. Hass, Susan D. Spencer, Beth E. Malloy, Austin L. Gurney, Steven A. Spencer, Walter C. Darbonne, William J. Henzel, Suzy C. Wong, Wun-Jing Kuang, Karl J. Oles\*, Bruce Hultgren, Lawrence A. Solberg Jr†, David V. Goeddel & Dan L. Eaton‡

The Departments of Molecular Biology, Cardiovascular Research and Protein Chemistry, Genentech, 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

\* Hematology Research Section, Mayo Clinic, Rochester, Minnesota 55905, USA

† Division Hematology-Oncology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, Florida 32224, USA

**Physiological platelet synthesis is thought to require the humoral activities of meg-CSF and thrombopoietin, which respectively promote proliferation and maturation of megakaryocytic cells. A meg-CSF/thrombopoietin-like protein that is present in plasma of irradiated pigs has been purified and cloned. This protein binds to and activates the c-mpl protein, a member of the cytokine receptor superfamily. The isolated Mpl ligand shares homology with erythropoietin and stimulates both megakaryocytopoiesis and thrombopoiesis.**

PLATELET production has long been thought to be regulated by lineage-specific humoral factors. Although several known haematopoietic growth factors and cytokines stimulate megakaryocytopoiesis and platelet production (reviewed in ref. 1), most are pleiotropic and consequently their roles in physiological regulation of thrombocytopoiesis are unclear. However, the plasma, serum and urine of thrombocytopenic animals and humans do contain megakaryocytopoietic and thrombopoietic activities<sup>2-13</sup> that are lineage-specific and distinct from known cytokines<sup>14-17</sup>. These activities are referred to as meg-CSF or thrombopoietin on the basis of their ability to affect either proliferation (meg-CSF) or maturation (thrombopoietin) of megakaryocytes<sup>18,19</sup>. Although these activities have been described since the 1960s, attempts to purify them have been unsuccessful.

Recently, the orphan cytokine receptor encoded by *c-mpl* (*c-Mpl*)<sup>20-23</sup> has been implicated in the regulation of megakaryocytopoiesis<sup>24</sup>. The expression of *c-mpl* appears to be restricted to primitive stem cells, megakaryocytes and platelets, indicating its downregulation during the differentiation of all haematopoietic lineages except megakaryocytes<sup>24</sup>. Moreover, *c-mpl* antisense oligonucleotides selectively inhibit megakaryocytic colony formation *in vitro* without affecting the growth of erythroid and granulomacrophage colonies<sup>24</sup>. These results suggest that a putative ligand for *c-Mpl* may be a megakaryocyte lineage-specific growth factor similar to the meg-CSF or thrombopoietin activities present in thrombocytopenic plasma.

Consistent with previous observations<sup>2-13</sup>, we have found that aplastic porcine plasma (APP) obtained from irradiated pigs stimulates human megakaryocytopoiesis *in vitro*. Here we report that this stimulatory activity is abrogated by the soluble extracellular domain of *c-Mpl*, identifying APP as a source of the putative Mpl ligand (ML). We have purified the ML from APP and used the amino-acid sequence information to isolate a human ML complementary DNA. The ML has sequence homology to erythropoietin and has both meg-CSF and thrombopoietin-like activities.

## Aplastic porcine plasma contains Mpl ligand

APP obtained from irradiated pigs stimulated human megakaryocytopoiesis *in vitro* (Fig. 1a). To determine whether

this stimulation was dependent on the putative ML, we examined whether a human Mpl-IgG fusion protein containing the extracellular domain of Mpl neutralized this activity. Mpl-IgG inhibited the megakaryocytopoietic activity of APP, whereas a control (natriuretic peptide receptor-IgG) fusion protein had no effect (Fig. 1a). This indicates that APP contains an ML, and that this ligand is necessary for the property of this plasma to stimulate megakaryocytopoiesis *in vitro*.

To facilitate purification of the ML, an Mpl-dependent cell proliferation assay was developed. Stable expression of several different growth factor receptors in factor-dependent cell lines confers responsiveness to the respective growth factor<sup>25-27</sup>. Because the cytoplasmic domain of *c-Mpl* can transduce a proliferation signal<sup>22,23</sup> and the extracellular domain of *c-Mpl* appeared to bind ML (Fig. 1a), we predicted that *c-Mpl* might be a single-chain receptor. We therefore expressed *c-mpl* in the murine interleukin-3 (IL-3)-dependent Ba/F3 cell line and tested the ability of APP to stimulate proliferation of the Ba/F3-*mpl* cells. Stable expression of human *c-mpl* indeed conferred APP responsiveness to Ba/F3-*mpl* cells cultured in the absence of IL-3 (Fig. 1b). This activity was completely abolished by soluble Mpl-IgG, but not by the control fusion protein (CD4-IgG). Normal porcine plasma did not stimulate the Ba/F3-*mpl* cells. These results indicate that APP contains a factor(s) that can bind the extracellular domain of Mpl and transduce a proliferative signal.

## Purification of the Mpl ligand from APP

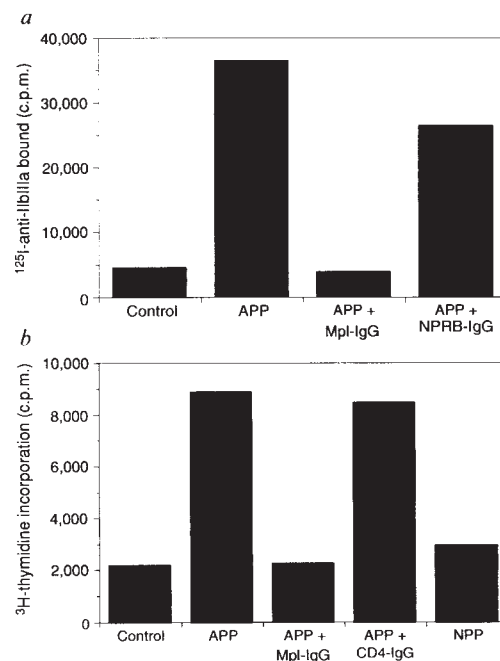
The ML was purified from APP using hydrophobic interaction, immobilized dye, and Mpl-affinity chromatography (Table 1). The activity present in column fractions was determined using the Ba/F3-*mpl* cell proliferation assay. The overall purification from 5 litres of APP was greater than  $4 \times 10^6$ -fold, with about 10% recovery of activity. We estimated the specific activity of the ligand eluted from the Mpl-affinity column to be  $\sim 3 \times 10^6$  units  $\text{mg}^{-1}$ .

Analysis of eluted fractions from the Mpl-affinity column by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of several proteins (Fig. 2a), the most intensely staining of which migrated at positions corresponding to  $M_r$  values of  $\sim 66\text{K}$ ,  $55\text{K}$ ,  $30\text{K}$ ,  $28\text{K}$  and  $14\text{K}$ . To determine the size of the ML, an aliquot of affinity-purified protein preparation was subjected to SDS-PAGE and resolved

‡ To whom correspondence should be addressed.

FIG. 1 Stimulation by APP and inhibition by Mpl-IgG of human *in vitro* megakaryocytopoiesis (a) and Ba/F3-*mpl* cell proliferation (b).

**METHODS.** Platelet-poor plasma was collected from normal or aplastic anaemic pigs. Pigs were rendered aplastic by irradiation with 900 cGy of total body irradiation using a 4 MeV linear accelerator. The irradiated pigs were supported for 6–8 days with intramuscular injections of cefazolin. Subsequently, their total blood volume was removed under general anaesthesia, heparinized, and centrifuged at 1,800g for 30 min to make platelet-poor plasma. The megakaryocyte-stimulating activity was found to peak 6 days after irradiation. a, Liquid suspension megakaryocytopoiesis assay: the effect of APP on human megakaryocytopoiesis was determined using a modification of the liquid suspension megakaryocytopoiesis assay described in ref. 43. Circulating peripheral blood progenitor cells (PSC) obtained from consenting patients were diluted fivefold with PBS and centrifuged at 800g for 15 min at room temperature. The cell pellets were resuspended in Iscove's modified Dulbecco's media (IMDM) and layered onto 60% percoll (density 1.077 gm ml<sup>-1</sup>, Pharmacia) and centrifuged at 800g for 20 min. The light-density mononuclear cells were collected at the interface and washed twice with IMDM and plated out at 1–2 × 10<sup>6</sup> cells per ml in IMDM containing 30% FBS (1 ml final volume) in 24-well tissue culture clusters (Costar). The cultures were incubated with or without 10% APP for 12–14 days in a humidified incubator at 37 °C in 5% CO<sub>2</sub> and air; 0.5 µg ml<sup>-1</sup> Mpl-IgG or natriuretic peptide receptor/IgG (NPRB-IgG<sup>44</sup>) was added at days 0, 2 and 4. After 12–14 days in culture, megakaryocytopoiesis was quantified using a radiolabelled murine IgG monoclonal antibody (HP1-1D) against the megakaryocyte-specific glycoprotein GPIIb/IIIa (provided by W. L. Nichols, Mayo Clinic) as described<sup>43</sup>. The average of duplicate determinations is shown. There was less than 10% variation between each duplicate. Mpl-IgG: a cDNA fragment encoding amino acids 1–491 of human Mpl was obtained by PCR from a human megakaryocytic CMK cell cDNA library and fused in-frame to a cDNA encoding the Fc region of human IgG1, as described<sup>44</sup>. The Mpl-IgG construct was subcloned into pRK5-tkneo and transfected into 293 human embryonic kidney cells by the calcium phosphate method<sup>45</sup>. The cells were selected in 0.4 mg ml<sup>-1</sup> G418 and individual clones were isolated. Mpl-IgG expression from isolated clones was determined using a human Fc-specific ELISA. The recombinant Mpl-IgG was purified on a protein A-Sepharose column (Pharmacia). b, Ba/F3-*mpl* cell proliferation assay: a DNA fragment corresponding to the entire coding sequence of *mpl* P (ref. 21) was obtained by PCR using as template cDNA prepared from the human megakaryocytic CMK cell line and was cloned into pRK5-tkneo. After linearization, this construct was introduced in Ba/F3 cells by electroporation (250 volts, 960 µF). Neomycin-resistant cells



were selected in 2 mg ml<sup>-1</sup> G418 and individual clones were obtained by limiting dilutions. Clones expressing *mpl* P were identified by FACS analysis using rabbit polyclonal antiserum raised against human Mpl-IgG. Stimulation of proliferation of Ba/F3-*mpl* cells in response to aplastic pig plasma was measured by the extent of <sup>3</sup>H-thymidine incorporation in the DNA. Cells were starved of IL-3 for 16 h then seeded in 96-well plates at a density of 25,000 cells per well in media containing APP at various concentrations in the presence or absence of Mpl-IgG or CD4-IgG<sup>42</sup> at 200 pM. After incubation for 22 h, 1 µCi <sup>3</sup>H-thymidine per well was added and the cells incubated for an additional 6 h before being collected. Incorporated radioactivity was determined in the presence of 40 µl of scintillation fluid (µrosint 20) using a Top Count Counter (Packard Instruments). The average of duplicate determinations is shown. There was less than 10% variation between each data point. One unit of activity produced 50% maximal stimulation.

proteins were eluted from gel slices and assayed (Fig. 2b). Most of the activity was found in the 28K–32K region of the gel, with a smaller amount of activity eluting in the 18K–20K region. The only proteins visible in these regions had *M<sub>r</sub>* values of 30K, 28K and 18K. To obtain sequence information on these proteins, the Mpl-affinity-purified preparation was separated by SDS-PAGE

and electroblotted onto polyvinylidene difluoride (PVDF)<sup>28</sup>. Amino-terminal sequence analysis<sup>29</sup> of these three proteins gave the identical sequence, SPAPPACDPRLLNKLLRDDHV-LHGRL (single-letter amino-acid code), indicating that these proteins are derived from a common precursor. Computer-assisted analysis showed this amino-acid sequence to be unique.

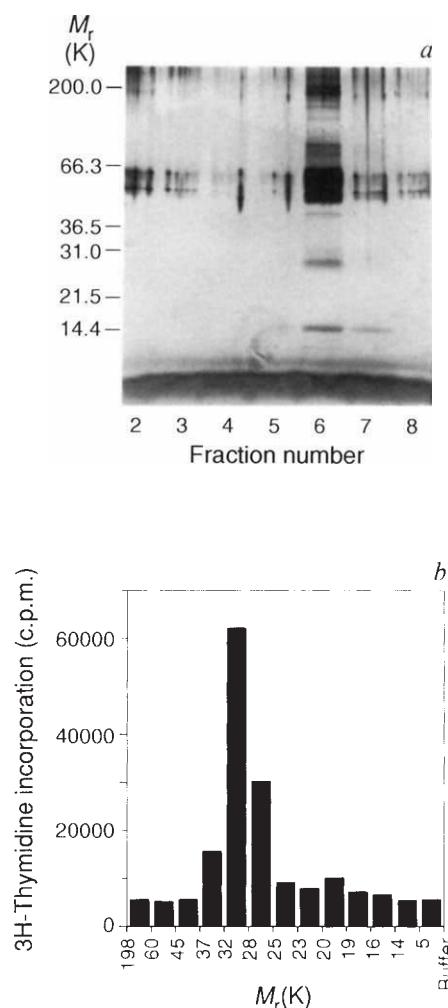
TABLE 1 Purification of the Mpl ligand from APP

| Sample | Volume (ml) | Protein (mg ml <sup>-1</sup> ) | Units ml <sup>-1</sup> | Units   | SA (Unit mg <sup>-1</sup> ) | Yield (%) | Fold purification     |
|--------|-------------|--------------------------------|------------------------|---------|-----------------------------|-----------|-----------------------|
| APP    | 5,000       | 50                             | 40                     | 200,000 | 0.8                         | —         | 1                     |
| Phenyl | 4,700       | 0.8                            | 40                     | 188,000 | 50                          | 94        | 62                    |
| Blue-S | 640         | 0.93                           | 400                    | 256,000 | 430                         | 128       | 538                   |
| Mpl-UL | 12          | 0.0005                         | 1,666                  | 20,000  | 3.3 × 10 <sup>6</sup>       | 10        | 4.1 × 10 <sup>6</sup> |

Aplastic porcine plasma obtained from irradiated pigs was made 4 M in NaCl and stirred for 30 min at room temp. The resultant precipitate was removed by centrifugation at 3,800 r.p.m. in a Sorvall RC3B and the supernatant was loaded onto a phenyl-Toyopearl column (220 ml) equilibrated in 10 mM sodium phosphate containing 4 M NaCl. The column was washed with this buffer until the absorbance at 280 nm was <0.05 and eluted with H<sub>2</sub>O. The eluted protein peak was diluted with H<sub>2</sub>O to a conductivity of 15 mS and loaded onto a blue-Sepharose column (240 ml) equilibrated in PBS. Subsequently, this column was washed with 5 column volumes each of PBS and 10 mM sodium phosphate containing 2 M urea. The column was eluted with 10 mM sodium phosphate containing 2 M urea and 1 M NaCl. The eluted protein peak was made 0.01% in octyl glucoside (*n*-octyl β-D-glucopyranoside) and 1 mM each in EDTA and Pefabloc (Boehringer Mannheim) and loaded directly onto tandemly linked CD4-IgG<sup>42</sup> and Mpl-IgG ultralink (Pierce) columns. The CD4-IgG (2 ml) column was removed after the sample was loaded and the Mpl-IgG (4 ml) column washed with 10 column volumes each of PBS and PBS containing 2 M NaCl, then eluted with 0.1 M glycine-HCl, pH 2.25. Fractions were collected into 0.1 volume of 1 M Tris, pH 8.0. The CD4-IgG and Mpl-IgG columns were made by coupling 10–20 mg of each to 0.5 g of Ultralink (UL) resin according to the manufacturer's instructions. A summary of the purification of the ML from 5 litres of APP is shown.



FIG. 2 Identification of ML by SDS-PAGE (a) and gel elution (b). **METHODS.** a, SDS-PAGE of purified ML. 200  $\mu$ l each of the eluted fractions 2 to 8 from the Mpl-affinity column were precipitated with acetone and solubilized in Laemmli-SDS-sample buffer. The sample was resolved on a 4–20% SDS-polyacrylamide gel (Novex) and proteins were visualized by silver staining. b, Elution of ML activity from an SDS gel. The affinity-purified ML (50  $\mu$ l) was resolved on a Novex 14% SDS-polyacrylamide gel run under non-reducing conditions. The sample was not heated. As a control, sample buffer without ligand was applied to a separate gel under identical conditions. Following electrophoresis the gel was cut into 12 slices, noting their position in relation to  $M_r$  markers. The 12 gel slices were placed into the cells in two Biorad model 422 electro-eluters and proteins were electro-eluted as previously described<sup>46</sup>. The eluted proteins from the 12 gel slices were dialysed overnight at 4 °C against PBS. Samples were then incubated on ice for 1 h. Precipitated SDS was removed by centrifugation in a microfuge. The supernatants were then again placed on ice for 1 h and microfuged. The final supernatants were diluted in PBS and assayed for Ba/F3-*mpl* cell proliferation activity.



Furthermore, this protein(s) is probably the ML because it co-elutes with activity from an SDS gel at two different regions along the gel.

### Molecular cloning of the Mpl ligand

On the basis of the N-terminal sequence obtained from the purified ligand, two degenerate oligonucleotide primer pools were used to amplify porcine genomic DNA by polymerase chain reaction (PCR). Assuming that the N terminus of the ML is encoded by a single exon, the correct amplification product should be 69 base pairs (bp) long. A fragment of the expected size was obtained and sequenced. The amino-acid sequence (PRLNKLRL) encoded between the PCR primers was identical to that obtained by N-terminal protein sequencing.

A synthetic oligonucleotide based on the sequence of the PCR fragment was used to screen a human genomic DNA library. Four clones with a similar restriction pattern were isolated and one of them was characterized further. A 390-bp *EcoRI*-*XbaI* fragment was found to contain a putative exon encoding 42 amino acids (Fig. 3a). The deduced amino-acid sequence varies by only five amino acids when compared to the first 26 amino acids of the porcine ML. The predicted amino-acid sequence immediately upstream of the sequence determined by direct protein sequencing is highly suggestive of a signal peptide. However, this sequence does not contain an initiation codon, suggesting the existence of an additional upstream exon.

To identify a source of ML messenger RNA for cDNA cloning reverse-transcribed PCR (RT-PCR) analysis was done on

mRNA prepared from various human fetal and adult tissues and cell lines, using as primers oligonucleotides corresponding to the ends of the exon sequence. A DNA fragment of the expected size (140 bp) was detected in adult kidney and fetal liver. A human fetal liver cDNA library ( $7 \times 10^6$  clones) in  $\lambda$ DR2 was screened with the oligonucleotide probe and four positive clones were isolated. The sequence of a clone with a 1.8-kilobase (kb) insert was determined.

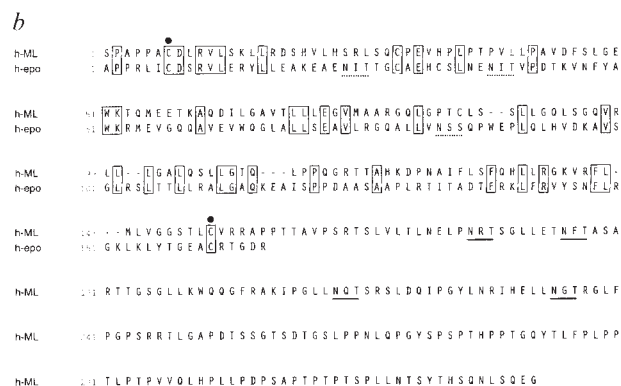
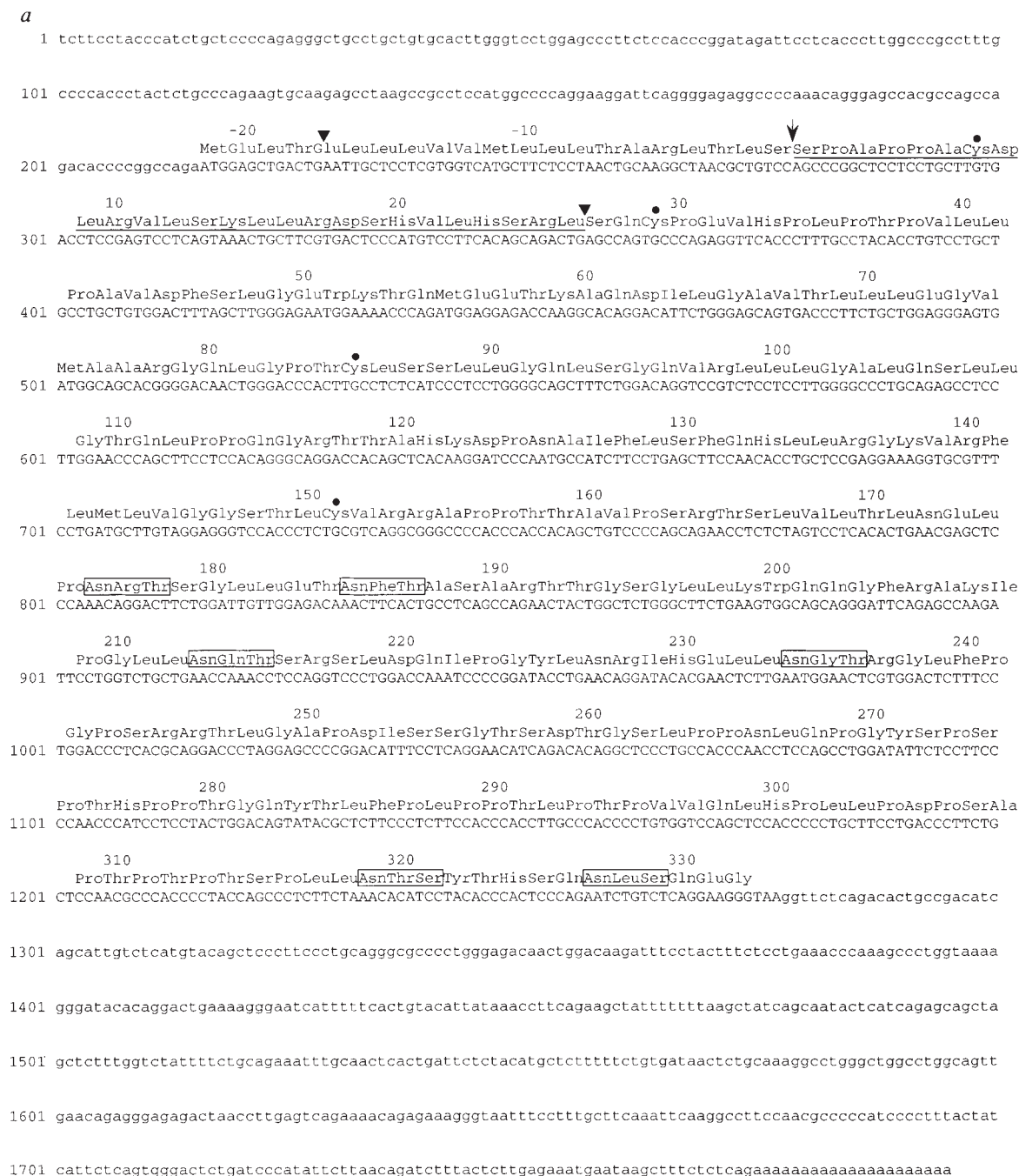
### Structure of the Mpl ligand

The human ML cDNA clone consists of 1,774 nucleotides followed by a poly(A)<sup>+</sup> tail (Fig. 3a). The presumed initiation codon at nucleotide position 216–218 is within a consensus sequence favourable for eukaryotic translation initiation<sup>30</sup>. It defines an open reading frame of 1,059 nucleotides, which predicts a primary translation product of 353 amino acids. Flanking this open reading frame are 215 nucleotides of 5' and 498 nucleotides of 3' untranslated sequence. The N terminus of the predicted amino-acid sequence is highly hydrophobic and probably represents a signal peptide. Computer analysis<sup>31</sup> indicates a potential cleavage site for signal peptidase between residues 21 and 22. Cleavage here would generate a mature polypeptide of 332 amino acids (38K) beginning with the N-terminal sequence obtained from protein sequencing of the porcine ML.

Comparison of the ML sequence with the Genbank sequence database revealed 23% identity between the N-terminal 153 residues of the ML and erythropoietin (Fig. 3b). When conservative substitutions are taken into account, this region of ML shows

FIG. 3 a. Nucleotide sequence and deduced amino-acid sequence of human ML cDNA. Nucleotides are numbered at the beginning of each line. Amino acids are numbered above the sequence starting at Ser 1 of the mature ML protein sequence. The 5' and 3' untranslated regions are indicated in lower case letters. The boundaries of the exon contained in the 390-bp *EcoRI*-*XbaI* fragment are indicated by arrows and the potential *N*-glycosylation sites are boxed. Cysteine residues are indicated by a dot above the sequence. The underlined sequence corresponds to the N-terminal sequence determined by protein sequencing of the pig ligand. b. Comparison of the ML and erythropoietin sequences. The predicted amino-acid sequence for the human ML is aligned with the human erythropoietin sequence<sup>34</sup>. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Potential *N*-glycosylation sites are underlined with a plain line for the ML and with a broken line for erythropoietin. The two cysteines important for erythropoietin activity are indicated by a dot.

**METHODS.** a. On the basis of the N-terminal sequence of the porcine ML, two primer pools were synthesized: *mpl.1* 5'-CCNGCNCNCNCNC-NGCNTGY-GA-3' (2,048-fold degenerate) and *mpl.2* 5'-NCCRTGNARNA-CRTGTRC-3' (2,048-fold degenerate). Porcine genomic DNA isolated from porcine peripheral blood lymphocytes was used as the template for PCR<sup>47</sup>. DNA (0.8 µg) was amplified in a 50 µl reaction containing 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 100 µg ml<sup>-1</sup> BSA, 400 µM dNTPs, 1 µM of each primer pool and 2.5 units of *Taq* polymerase. Initial template denaturation was at 94 °C for 8 min, followed by 35 cycles of 45 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The final cycle was allowed to extend for 10 min at 72 °C. A 69-bp product was obtained and subcloned in pGEMt and three clones were sequenced. On the basis of the sequence obtained, a 45-mer deoxyoligonucleotide called pR45 was designed and synthesized: 5'-GCCGTGAAGGACGTGGTGCACGAAGCAGTTATTTAGGAGTCG-3'. The oligonucleotide was labelled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase and used to screen a human genomic library in  $\lambda$ Gem12 (provided by U. Schindler) under low-stringency hybridization conditions<sup>48</sup>. Positive clones were isolated and a 390-bp *EcoRI*-*XbaI* fragment hybridizing to the probe was cloned in pBluescript SK- and sequenced. On the basis of this sequence, two oligonucleotides were synthesized corresponding to the ends of the predicted exon sequence: forward primer: 5'-AGAAATGTCTCTCGTGGTCATGCTCT-3' and reverse primer: 5'-CAGTCTGCCGTGAAGGACATGG-3'. These oligonucleotides were used to prime a PCR reaction using the conditions described above. cDNAs from various human tissues and cell lines were used as a template. The expected 140-bp product was identified in fetal liver cDNA. A fetal liver cDNA library in  $\lambda$ DR2 was screened with the pR45 oligonucleotide as described above. Positive clones were isolated and characterized. The 1.8-kb insert of clone FL2b was subcloned in M13 and both strands were sequenced by standard fluorescent dye terminator and dye primer methods on an ABI373 automated sequencer.





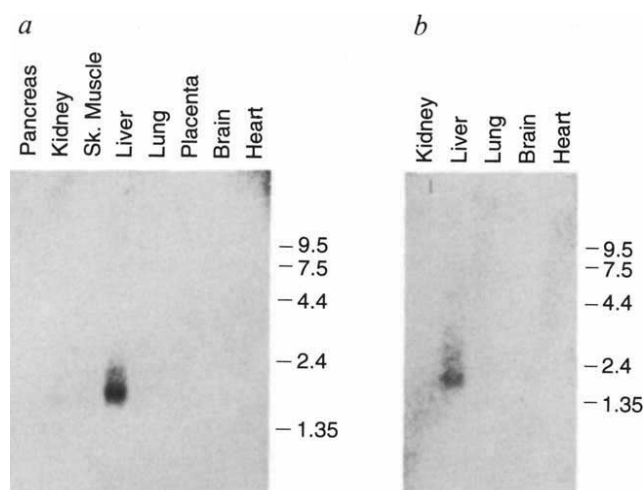


FIG. 4 Northern blot analysis of various human adult (a) or fetal tissues (b). Northern blots of  $\sim 2 \mu\text{g}$  poly(A)<sup>+</sup> RNA from various human tissues were purchased from Clontech. The membranes were hybridized overnight with a single-stranded DNA probe corresponding to position 219–470 of the human ML. The probe was generated by asymmetric PCR in the presence of  $^{32}\text{P}$ -dATP. The blot was washed at  $42^\circ\text{C}$  in  $0.2 \times \text{SSC}$ , 0.1% SDS and then exposed for 3 days to a storage phosphor imaging plate.

50% similarity to erythropoietin. Both erythropoietin and the ML contain four cysteines, three of which are conserved. Site-directed mutagenesis experiments have shown that the first and last cysteines of erythropoietin form a disulphide bond essential for function<sup>32</sup>. By analogy, the first and last cysteines of ML may also form a critical disulphide bond. None of the three glycosylation sites within erythropoietin are conserved in the ML. The six potential *N*-linked glycosylation sites predicted by ML sequence are all located in the C-terminal half of the protein.

Like erythropoietin, the ML mRNA does not contain the consensus polyadenylation sequence AAUAAA, nor the regulatory element AUUUA that is present in 3' untranslated regions of many cytokines and is thought to influence mRNA stability<sup>33</sup>. Northern blot analysis reveals low levels of a single 1.8-kb ML RNA transcript in both fetal and adult liver (Fig. 4). After longer exposure, a weaker band of the same size could be detected in adult kidney (not shown). By comparison, erythropoietin is expressed in fetal liver and, in response to hypoxia, the adult kidney and liver<sup>34,35</sup>.

The importance of the C-terminal region of the ML remains to be elucidated. On the basis of the presence of the six potential sites for *N*-linked glycosylation and the ability of the ligand to bind lectin-affinity columns (data not shown), this region of the ML is probably glycosylated. In some gel-elution experiments we observed activity resolving with  $M_r \sim 60\text{K}$  (data not shown), which may represent the full-length, glycosylated molecule. The C-terminal region may therefore act to stabilize and increase the half-life of circulating ML. In the case of erythropoietin, the non-glycosylated form has full *in vitro* biological activity, but has a significantly reduced plasma half-life relative to glycosylated erythropoietin<sup>36–38</sup>. The C-terminal domain of ML contains two di-basic amino-acid sequences (Arg-Arg motifs at positions 153–154 and 245–246; Fig. 3a) that could serve as potential processing sites. Cleavage at these sites may be responsible for generating the 30K, 28K and 18K forms of the ML isolated from APP. Importantly, the Arg<sub>153</sub>-Arg<sub>154</sub> sequence occurs immediately following the erythropoietin-like domain of the ML.

The observations indicate that full-length ML may represent a precursor protein that undergoes limited proteolysis to generate the mature ligand. Interestingly, a comparison of human and porcine ML sequences shows 83% identity between the erythropoietin-like domains, but only 67% between the C-terminal

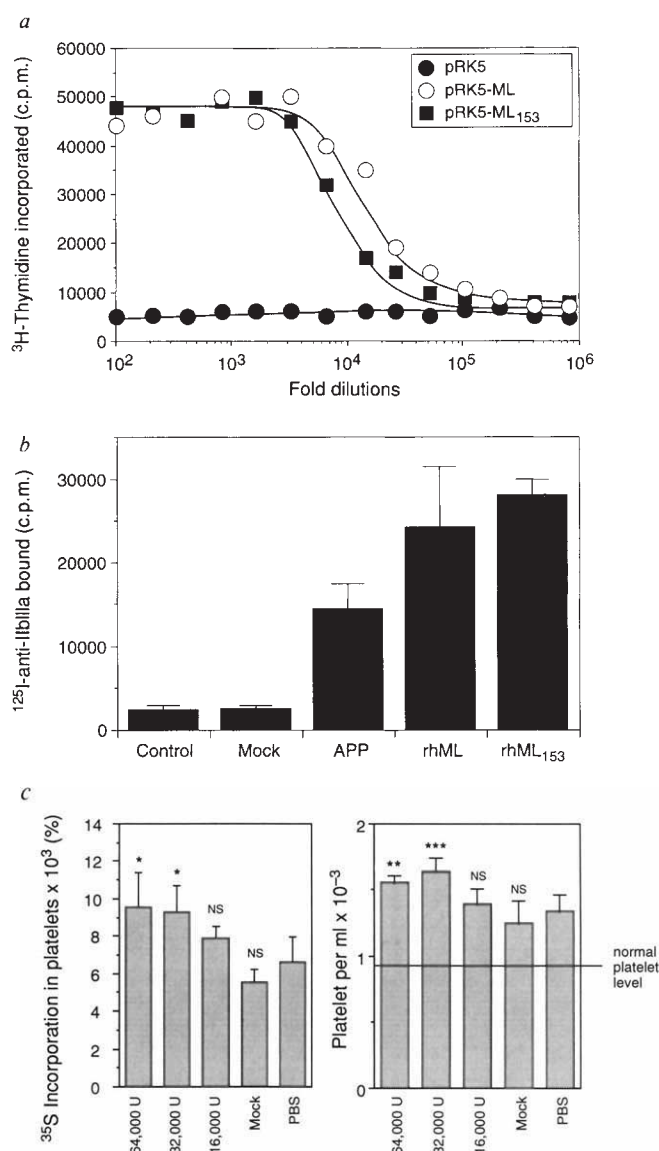


FIG. 5 Effect of rhML on Ba/F3-*mpl* cell proliferation (a), *in vitro* human megakaryocytopoiesis (b), and murine thrombopoiesis (c).

**METHODS.** pRK5-ML<sub>153</sub> was generated by introducing a stop codon after residue 153 by PCR. 293 cells were transfected with either pRK5-hML or pRK5-ML<sub>153</sub> by the calcium phosphate method<sup>45</sup>. Media were then conditioned for 36 h and tested for activity in the Ba/F3-*mpl* cell proliferation (a) and *in vitro* megakaryocytopoiesis (b) assays described in Fig. 1. The effect of partially purified rhML on platelet production *in vivo* (c) was determined using the rebound thrombocytosis assay described in ref. 39. Briefly, mice were given a single injection of goat anti-mouse platelet serum (day 0) and on day 5 and 6, during rebound thrombocytosis, the mice were injected with test samples or excipient. On day seven,  $30 \mu\text{Ci Na}_2^{35}\text{SO}_4$  was injected and 24 h later  $^{35}\text{S}$  incorporation into circulating platelets and platelet counts were determined. Partially purified rhML was prepared from 200 ml conditioned media obtained from 293 cells transfected with pRK5-hML. The media were passed through a 2 ml blue-Sepharose column equilibrated in PBS and the column was washed with PBS and eluted with PBS containing 2 M each of urea and NaCl. The active fraction was dialysed into PBS and made  $1 \text{ mg ml}^{-1}$  with endotoxin-free BSA. Medium from mock-transfected cells was treated similarly. The sample contained less than one unit of endotoxin per ml (Limulus Amebocyte Assay, Bio Whittaker). Mice were injected with either 64,000, 32,000 or 16,000 units of rhML, a mock preparation, or excipient alone. Each group consisted of six mice. The mean and standard deviation of each group is shown. *P* values were determined by a 2-tailed *t*-test comparing medians; \* *P* = 0.003; \*\* *P* = 0.0005; \*\*\* *P* = 0.0001; NS, not significant.

domains. The dibasic site present at position 153–154 in the human ML is conserved in porcine ML (unpublished results), consistent with the possibility that the erythropoietin-like domain of the ML represents the mature ligand.

### Expression of recombinant *mpl* ligand

To confirm that the cloned cDNA encoded a ligand for Mpl, it was expressed in mammalian cells under the control of the cytomegalovirus immediate-early promoter using the expression vector pRK5-hML. Supernatants from transiently transfected human embryonic kidney 293 cells were found to stimulate <sup>3</sup>H-thymidine incorporation in Ba/F3-*mpl* cells, but not in parental Ba/F3 cells (Fig. 5a). Media from 293 cells transfected with the pRK vector alone did not contain this activity. Addition of Mpl-IgG to the media abolished the stimulation (data not shown). These results show that the cloned cDNA encodes a functional human ML (hML).

To determine whether the erythropoietin-like domain alone can bind and activate Mpl, a truncated form of hML consisting of residues 1–153 (rhML<sub>153</sub>) was expressed in 293 cells. Supernatants from transfected cells have activity similar to that in supernatants from cells expressing the full-length hML (Fig. 5a), indicating that the C-terminal domain of ML is not required for binding and activation of c-Mpl.

### Stimulation by the *mpl* ligand

Both the full-length (rhML) and the truncated (rhML<sub>153</sub>) forms of recombinant hML stimulated human megakaryocytopoiesis *in vitro* (Fig. 5b). Whether this stimulation is caused by direct action of ML or requires a serum or induced cellular factor(s) cannot be ruled out. However, this effect was observed in the absence of other exogenously added haematopoietic growth factors. With the exception of IL-3, the ML is the only haematopoietic growth factor that exhibited this activity. IL-11, IL-6, IL-1, erythropoietin, G-CSF, IL-9, LIF, Kit ligand, m-CSF, OSM and GM-CSF had no effect on megakaryocytopoiesis when tested separately in our assay (data not shown). This result demonstrates that the ML has megakaryocyte-stimulating activity, and indicates a role for ML in regulating megakaryocytopoiesis.

Thrombopoietic activities present in plasma of thrombocytopenic animals stimulate platelet production in a mouse rebound thrombocytosis assay<sup>39,40</sup>. In this model, mice are made acutely thrombocytopenic using specific antiplatelet serum, resulting in a predictable rebound thrombocytosis. Such immunothrom-

bocythaemic mice are more responsive to exogenous thrombopoietin-like activities than are normal mice<sup>39</sup>, just as exhypoxic mice are more sensitive to erythropoietin than are normal mice<sup>41</sup>. To determine whether the rML stimulates platelet production *in vivo*, mice in rebound thrombocytosis were injected with partially purified rhML. Platelet counts and incorporation of <sup>35</sup>S into platelets were then quantified. Injection of mice with 64,000 or 32,000 units of rML significantly increased platelet production, as shown by a ~20% increase in platelet counts ( $P=0.0005$  and  $0.0001$ , respectively) and a ~40% increase in <sup>35</sup>S incorporation into platelets ( $P=0.003$ ) in the treated mice versus control mice injected with excipient alone (Fig. 5c). This level of stimulation is comparable to that observed with IL-6 in this model (data not shown). Treatment with 16,000 units of rML or a preparation from mock-transfected cells did not significantly stimulate platelet production. These results indicate that ML stimulates platelet production in a dose-dependent manner and therefore possesses thrombopoietin-like activity.

### Megakaryocytopoiesis and the *mpl*-ligand

It has been proposed that megakaryocytopoiesis is regulated at multiple cellular levels<sup>18,19</sup>. This is based largely on the observation that certain haematopoietic growth factors stimulate proliferation of megakaryocyte progenitors, whereas others appear primarily to affect maturation<sup>1</sup>. Our results, together with previous findings, suggest that the ML acts both as an proliferative and maturation factor. Although the *in vitro* megakaryocytopoiesis assay described here does not differentiate between activities that affect either proliferation or maturation, several lines of evidence suggest that ML stimulates proliferation of megakaryocyte progenitors. APP stimulates both proliferation and maturation of human megakaryocytes *in vitro* (L.A.S., unpublished data), and this stimulation is completely inhibited by Mpl-IgG (Fig. 1a). Furthermore, the inhibition of megakaryocyte colony formation by *c-mpl* antisense oligonucleotides<sup>24</sup>, and the finding that *c-mpl* can transduce a proliferative signal in cells into which it is transfected<sup>22,23</sup>, also indicate that ML stimulates proliferation. The apparent expression of *c-mpl* during all stages of megakaryocyte differentiation<sup>24</sup> and the ability of recombinant ML to stimulate platelet production rapidly *in vivo* indicate that ML also affects maturation. The availability of recombinant ML makes possible a careful evaluation of its role in regulating megakaryocytopoiesis and thrombopoiesis, as well as its potential to influence other haematopoietic lineages. □

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# Dust formation in Nova Cassiopeiae 1993 seen by ultraviolet absorption

S. N. Shore\*, S. Starrfield†, R. Gonzalez-Riestra‡, P. H. Hauschildt† & G. Sonneborn§

\* Department of Physics and Astronomy, Indiana University South Bend, 1700 Mishawaka Avenue, South Bend, Indiana 46634-7111, USA

† Department of Physics and Astronomy, Arizona State University, Box 871504, Tempe, Arizona 85287-1504, USA

‡ Astrophysics Division, Space Science Department, ESTEC and International Ultraviolet Explorer, European Space Agency, Villafranca, Spain

§ Laboratory for Astronomy and Solar Physics, Code 681, NASA Goddard Space Flight Center, Greenbelt, Maryland 20771, USA

THE clouds of gas in interstellar space also contain grains of dust, whose properties and origins have been the focus of debate for decades. Some dust formation has been assumed to take place in novae explosions<sup>1–5</sup>, as was first implied by the observation of a steep decrease in the amount of light emitted by the nova<sup>1,2</sup> DQ Herculis 1934 about 100 days after outburst, presumed to be due to extinction by dust. Here we report observations from the International Ultraviolet Explorer satellite which show directly the onset of dust formation in Nova Cassiopeiae 1993, a classical nova of the same type as DQ Her 1934. The dust formed very quickly—about 70 days after the nova explosion—despite the initially high temperature of the ejecta. Our results suggest that high-energy photons are absorbed efficiently by the gas in the ejecta, lowering the temperature in the gas while it is still dense, and thereby allowing molecules to form and then to condense into dust.

Our observations of the ultraviolet evolution of Nova Cas 1993 began shortly after its discovery was announced on 1993 December 12 and were continued until 1994 February 21.3 UT. They were terminated because of power-related pointing constraints on the International Ultraviolet Explorer (IUE) spacecraft. The ultraviolet light curve is shown in Fig. 1b. Here we discuss only a small portion of the data covering the period just before and during the optical transition.

The optical light curve of Nova Cas 1993 (Fig. 1a) closely resembles that of DQ Her 1934. After discovery on 1993 December 7 (ref. 6), it displayed some flaring activity superimposed on a slow decline of  $\sim 2$  mag (Fig. 1) until about 1994 February 13. Around this date the decline accelerated, and during the next two weeks its optical brightness dropped to below  $V = 15$  mag.

We obtained a set of IUE spectra on February 10.5 UT (JD 2449394.0). The 1,200–2,000 Å spectrum is shown in Fig. 2a. Our next observation was obtained on February 18.2 UT (JD 2449401.7), by which time the brightness of the nova had fallen to  $V \approx 10.5$  mag; the spectrum is displayed in Fig. 2b. The entire spectrum is depressed in flux almost uniformly by a factor of five. There is no evidence of any rise in the ultraviolet extinction toward shorter wavelengths, as is observed for normal interstellar extinction. The 2,200–3,400 Å spectrum was also depressed by a factor of five compared with the long-wavelength spectrum obtained on 10 February.

The extinction curve was obtained by taking a ratio of the 21 February spectra to those obtained on 10 February. Figure 3a shows the extinction curve that would have resulted from obscuration of the nova by if it had the same size and opacity distribution as interstellar grains<sup>7,8</sup>. The dust formed by Nova Cas 1993 is clearly very different (Fig. 3b). The nearly grey extinction at wavelengths shorter than Mg II 2,800 Å can best be explained by a population of very large grains with an optical depth of  $\sim 4$ .

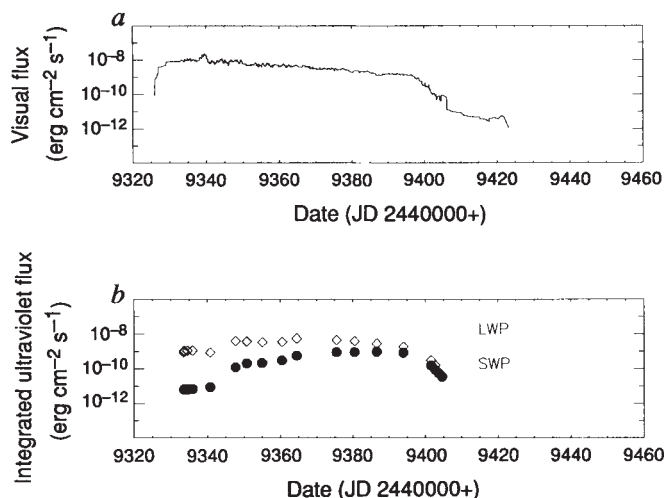


FIG. 1 a, Optical light curve for Nova Cas 1993 (Y. Takakeda, personal communication). b, Ultraviolet light curve from low-resolution IUE spectra. The two ultraviolet curves cover separate wavelength intervals: SWP, 1,200–2,000 Å; LWP, 2,200–3,400 Å. No extinction correction has been applied to these data.

In order to produce a flat absorption spanning a factor of three in wavelength in the ultraviolet, the grains must have sizes in excess of  $2\pi a/\lambda > 4$  (ref. 9), where  $a$  is the grain radius and  $\lambda$  is the wavelength, so that the grains must be bigger than  $\sim 0.2 \mu\text{m}$ . An optical depth of unity implies that the total mass of dust is  $\sim 10^{-7} Q_{\text{abs}} \rho_{\text{dust}} M_{\odot}$ , where  $Q_{\text{abs}}$  is the absorption efficiency ( $\sim 1$  for large grains<sup>9</sup>),  $\rho_{\text{dust}}$  is the mass density of the dust (in this case assumed to be  $\sim 3 \text{ g cm}^{-3}$  for silicates) and  $M_{\odot}$  is the solar mass. This dust mass is comparable with values obtained from infrared studies of other novae, especially NQ Vulpeculae, (ref. 1) by a completely independent method. These results significantly strengthen the conclusions, drawn from the infrared observations, that large particles of warm dust forms quickly in the ejecta of novae of the DQ Her type<sup>4,5</sup>.

The flux removed from the ultraviolet will heat any circumstellar grain population, and the initial stages of the extinction event should have produced a rapid rise in the infrared emission. The foreground interstellar extinction appears to be of the order of  $E_B - V = 0.5$ . This is inferred from model atmosphere fitting (see below) and also from the requirement that the bolometric luminosity remain constant during at least the optically thick phase

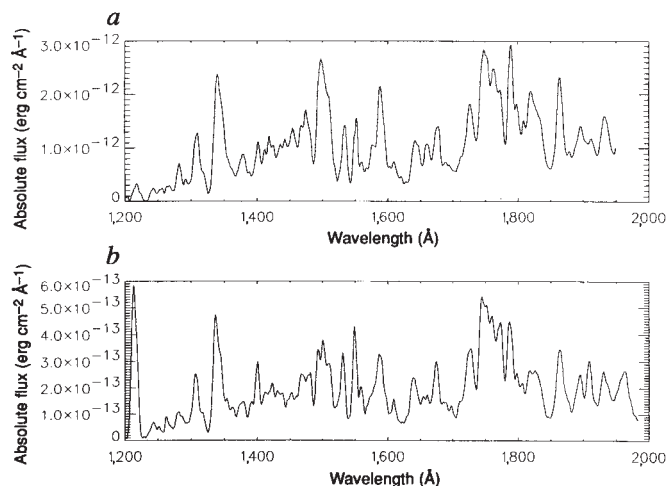


FIG. 2 The observed low-resolution short-wavelength IUE spectra obtained before and during the dust forming event. a, February 10.5 UT; b, February 21 UT. Note the change in the flux levels.

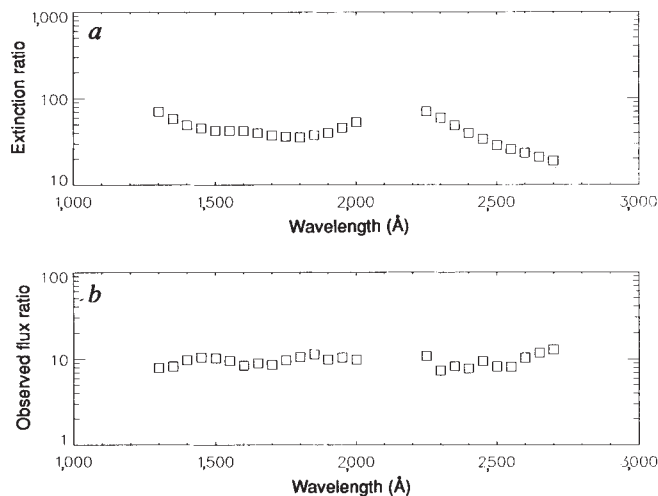


FIG. 3 Comparison of IUE spectra taken on February 10.5 ut (JD 244940.0) with those taken during the transition on February 18.2 ut (JD 244940.6). The spectra have been binned to 50-Å resolution. The region between 2,000 and 2,350 Å has high noise and has been excluded. Wavelengths longer than 2,800 Å are contaminated with scattered sunlight and have also been excised. *a*, Extinction curve assuming normal interstellar grain mixture for the IUE ultraviolet spectral range. The absolute value of the extinction depends on the visual scaling, so only the trend of the curve is important. *b*, Derived extinction law for Nova Cas 1993 (see text). The data have been binned to 50-Å intervals. The interstellar extinction does not affect this method because it is extrinsic to the source and cancels on taking ratios of spectra.

of the outburst. We have previously used this procedure for several novae, notably V1974 Cygni (ref. 10), and it appears to hold from infrared spectra as well<sup>1</sup>. We have used this extinction to derive the bolometric flux at the last stage before the minimum (Fig. 4). The maximum integrated infrared flux is therefore predicted to be  $\sim 8 \times 10^{-8} \text{ erg cm}^{-2} \text{ s}^{-1}$ , assuming that the grains completely reradiate the absorbed ultraviolet flux. This agrees with observations<sup>11</sup>. The observed dust temperature was  $\sim 1,200 \text{ K}$  on 15 February (ref. 12).

We emphasize that the dust temperature should not decrease monotonically with time because the absorption-line opacity

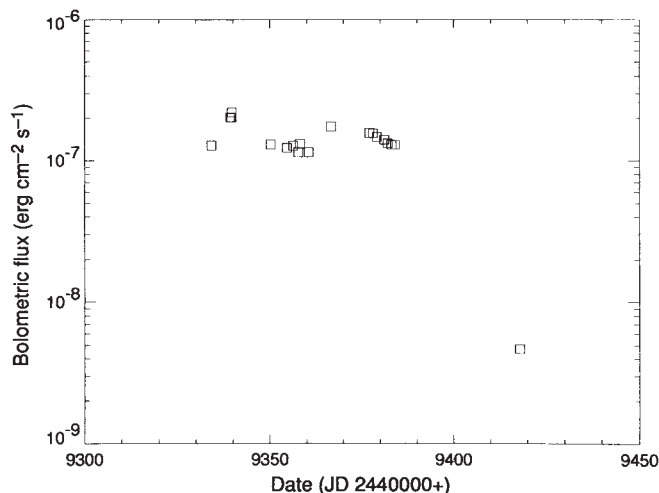


FIG. 4 The bolometric light variations of Nova Cas 1993, derived using optical and ultraviolet data. The interstellar extinction was assumed to be  $E(B-V) = 0.5$  (ref. 12). The dramatic drop after JD 2449395 is due to circumstellar dust obscuration. The bolometric magnitude is probably constant after the start of the transition, and the flux deficit accounts for the observed infrared emission after the start of the transition (see text).

decreases as the shell expands, thus increasing the ultraviolet heating of the grains faster than the expansion reduces their illumination by the central star. Grain-size distributions inferred from the infrared emissivity are therefore harder to interpret than our absorption measurements<sup>1</sup>. Additional support for ultraviolet heating is provided by the diffuse emission features at 3.4  $\mu\text{m}$  wavelength that were reported on 10 January (ref. 13). The emission is thought to be produced by ultraviolet irradiation of polycyclic aromatic hydrocarbons that may have been present during the period of formation of the larger grains.

Model atmosphere fitting to the pre-transition spectrum yields an effective temperature of the pseudo-photosphere of  $\sim 26,000 \text{ K}$  (ref. 14). With a maximum velocity of  $1,600 \text{ km s}^{-1}$ , the radius of the shell at  $\sim 70 \text{ d}$  into the outburst,  $9 \times 10^{14} \text{ cm}$ , yields a probable radius for the central source of the order of  $10^{12} \text{ cm}$  (solar radii) assuming a dust temperature of  $1,200 \text{ K}$ . This is close to the predicted radius of the rekindled white dwarf at the time when it is in equilibrium nuclear burning<sup>15</sup>. If the distance is of order 4 kpc (ref. 16), then the maximum luminosity is  $\sim 60,000 L_{\odot}$  (where  $L_{\odot}$  is the solar luminosity), corresponding to the Eddington luminosity for a white dwarf of  $\sim 1 M_{\odot}$ .

We find that the ultraviolet spectrum of Nova Cas 1993 immediately before the onset of dust formation is modelled better by a stellar wind than by free expanding ejecta, in contrast to recent novae such as V1974 Cyg<sup>14</sup>. The presence of pseudo-P-Cyg profiles in the ultraviolet during the early stages of the outburst, coupled with the extreme saturation of the overlapping atomic absorption lines and the absence of spectral windows, are reproduced by a wind outflow. In addition, high-resolution IUE spectra taken in the early outburst display many relatively narrow saturated iron peak absorption lines; the widths of these lines indicate that they are formed in a part of the outflow that has a very small velocity gradient, less than a few hundred kilometres per second. Models of the optically thick stage of V1974 Cyg and other novae do not show such regions. This dense spectral distribution of absorbers, also called the 'iron curtain', produces a large opacity that leads to a low temperature for the outer portions of the ejecta because of a reduction in the ultraviolet irradiation of the material at large distance.

Dust should form in a wind when a large mass fraction of this wind reaches an outer radius of the order of  $10^{14} - 10^{15} \text{ cm}$  during the optically thick phase of the 'iron curtain', at this stage, the temperature is low enough (below the evaporation temperature for astrophysical silicates and graphite,  $\leq 1,200 \text{ K}$ ) for grains to coalesce and remain stable. The mass at large distances is higher in the terminal velocity region of a wind than in the outermost portions of an explosive ejection because of its constant expansion velocity and shallower density profile. The detection of CO emission on 13 December (ref. 17) supports the conclusion that a large mass of cold gas was present at large distances, even in the earliest stages of the outburst. A wind also explains the comparatively sharp absorption features observed in the high-resolution 2,200–3,300 Å IUE spectra during the first 2 months of outburst<sup>14</sup>.

The coincidence of the 'iron curtain' stage of a steady-state outflow with dust formation in Nova Cas 1993 also has implications for the luminous blue variable stars. These are massive stars that evolve very near their Eddington luminosities and undergo massive wind phases, when as much as  $10^{-2} M_{\odot} \text{ yr}^{-1}$  is ejected<sup>18</sup>. The most luminous, such as AG Carinae and  $\eta$  Carinae, are imbedded in dusty circumstellar nebulae, even though their gaseous environments are completely ionized. The dust formed in Nova Cas 1993 is like that inferred for AG Car, where the grain sizes derived from ultraviolet spectra of the circumstellar reflection nebula are also large<sup>19,21</sup>. The dust in this luminous blue variable probably forms under similar circumstances, in the outer regions of a very opaque stellar wind. The origin of this dust has been problematic because the observed pseudo-photospheric temperatures for the luminous blue variables are never lower than  $\sim 7,000 \text{ K}$ , about the temperature of an A-type supergiant.



Davidson<sup>22</sup> has described how continuum opacities limit the spectroscopic excursions of these stars during outburst. The Nova Cas 1993 formation consequently illustrates how such an environment can succeed in forming dust. Once enough material has reached large distances, at high enough densities, the grains coalesce. □

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## Optical measurements of the superconducting gap in single-crystal $K_3C_{60}$ and $Rb_3C_{60}$

L. Degiorgi\*, G. Briceno†, M. S. Fuhrer†, A. Zettl† & P. Wachter\*

\* Laboratorium für Festkörperphysik ETH-Zürich, CH-8093 Zürich, Switzerland

† Department of Physics, University of California at Berkeley, Berkeley, California 94720, USA

THE mechanism of superconductivity in alkali-metal compounds of  $C_{60}$  (refs 1, 2) remains controversial. Electron-pairing mechanisms based on electron-phonon coupling involving both low-frequency (intermolecular) vibrations<sup>3</sup> and high-frequency (intramolecular) modes<sup>4,5</sup>—the strong-coupling and weak-coupling cases respectively—have been proposed. These two mechanisms have different associated energy scales, which is reflected in the magnitude of the superconducting energy gap. Measurements of the gap for these compounds have been reported previously for powder samples<sup>6–8</sup>, but are somewhat discrepant or ambiguous, perhaps owing to grain-size or grain-junction effects. Here we report measurements on large single-crystal samples of  $K_3C_{60}$  and  $Rb_3C_{60}$  using optical reflectivity. We obtain values for the reduced gap ratio,  $2\Delta/k_B T_c$ , of 3.44 and 3.45 respectively, consistent with predictions for a mechanism based on standard Bardeen-Cooper-Schrieffer (BCS) electron-phonon coupling to intramolecular modes.

High-quality single crystals of  $K_3C_{60}$  and  $Rb_3C_{60}$  were prepared by doping vapour-transport-grown  $C_{60}$  crystals with alkali metals. The synthesis follows a method similar to that described previously<sup>9,10</sup>. The crystals had a superconducting transition

temperature  $T_c$  of 20.3 and 30.5 K (that is, where the resistivity  $\rho(T_c)=0$  (refs 9, 10)), and had large and shiny surfaces with dimensions  $1.5 \times 2$  mm and  $2 \times 2$  mm for  $K_3C_{60}$  and  $Rb_3C_{60}$ , respectively.

Reflectivity measurements ( $R(\nu)$ ) were performed between 2 meV and 6 eV as a function of temperature using three different spectrometers, with overlapping frequency ranges as described in previous work<sup>8</sup>. The absolute accuracy of the measurement is  $\sim 0.8\%$ , whereas the relative accuracy (important for the temperature dependence of  $R(\nu)$ ) is better than 0.3%.

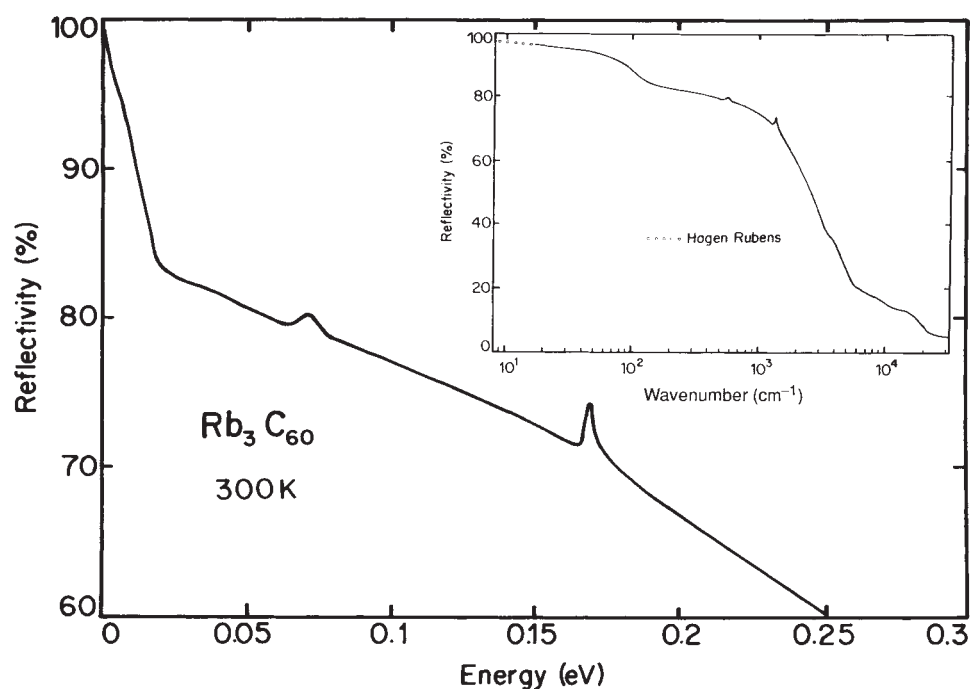
The reflectivity of  $Rb_3C_{60}$  in the normal state is displayed in Fig. 1 (results for  $K_3C_{60}$  are similar). Besides the overall metallic behaviour (see inset and ref. 8), we recognize in the mid-infrared frequency range two well defined absorptions at 0.07 and 0.17 eV, which are ascribed to the infrared-active  $T_{1u}$  phonon modes<sup>11,12</sup>. These modes were missing in our previous investigation of the pressed-pellet specimens (probably because of the large surface scattering due to the grains)<sup>8</sup>. Their appearance and the fair agreement with the expected frequency of these modes for this alkali-metal doping level (that is,  $A_xC_{60}$  with  $x=3$ )<sup>11,12</sup> are evidence for the very good quality of the samples and the outstanding resolution of these new optical measurements.

Figure 2 presents the reflectivity spectra in the far-infrared of  $K_3C_{60}$  and  $Rb_3C_{60}$  single crystals. It is clearly seen that below 0.014 eV,  $R(\nu)$  is enhanced and the onset of this BCS-like enhancement is coincident with  $T_c$ . At the lowest temperature ( $\sim 6$  K) the reflectivity is, within experimental error, 100% below a threshold frequency of  $\sim 6$  and 9 meV for  $K_3C_{60}$  and  $Rb_3C_{60}$ , respectively. By performing Kramers-Kronig transformations<sup>8</sup>, we obtain the optical conductivity  $\sigma_1(\nu)$ , which is displayed in Fig. 3 at several temperatures. The optical conductivity at 6 K is zero up to the threshold frequencies indicated above, which we ascribe to the optical identification of the superconducting gap. These values correspond to a reduced gap ratio of  $2\Delta/k_B T_c = 3.44$  and 3.45 for  $K_3C_{60}$  and  $Rb_3C_{60}$ , respectively, which suggests a weak coupling BCS limit. Our earlier measurements<sup>8</sup> on a pressed pellet hinted at this conclusion; but scattering due to grain-size effects may play an important role in polycrystalline samples, reducing or partially smearing out the intrinsic gap value.

The normal-state properties (spectra above  $T_c$  in Fig. 3) deviate considerably from the simple Drude behaviour expected for conventional metals (that is,  $\sigma_1(\nu)$  should be constant in the frequency range of Fig. 3), as discussed in ref. 8. This is due to a broad mid-infrared absorption at  $\sim 60$  meV, which produces a shallow minimum in  $\sigma_1(\nu)$  around 12 meV. The presence of such a mid-infrared absorption might lead to another explanation of the data: that the behaviour of  $R(\nu)$  below  $T_c$  is due to a type of plasma-edge effect<sup>13</sup>. Both the coincidence with  $T_c$  of the onset of the temperature dependence of  $R(\nu)$ , and particularly the absence of any zero crossing of the real part of the dielectric function  $\epsilon_1(\nu)$  (which would define the screened plasma frequency)<sup>13</sup> in the far-infrared frequency range, clearly rule out this explanation.

The experimental electrodynamic response is in a fairly good agreement with the Mattis-Bardeen calculation within the BCS framework<sup>14</sup> or, in a more sophisticated way, with the predictions of the standard Eliashberg theory, as discussed previously for polycrystalline specimens<sup>8</sup>. Within the Eliashberg formalism, one considers a realistic phonon spectrum and directly singles out those phonon modes that are relevant for superconductivity. This approach reduces to the weak coupling BCS Mattis-Bardeen calculation in the limit of the average phonon frequency being much greater than  $T_c$ , but impurity scattering effects can also be incorporated. We found that the self-consistent Eliashberg calculation within a phonon model spectrum dominated by high-frequency intramolecular modes gives a reduced gap ratio of  $\sim 3.5$  and moreover reproduces perfectly the experimental dynamical conductivity<sup>8</sup>. On the contrary, an important disagreement is found when low-energy

FIG. 1 Normal state reflectivity spectrum of  $\text{Rb}_3\text{C}_{60}$  in the mid-infrared frequency range. The inset displays the whole spectrum (note the logarithmic scale). The Hagen Rubens extrapolation of the reflectivity data to zero frequency corresponds to the expression  $R(\nu) = 1 - 2(\nu/\sigma_0)^{1/2}$ , where  $\sigma_0$  is the d.c.-conductivity.



phonon modes are considered; then,  $2\Delta/k_B T_c$  turns out to be of the order of 5. This is consistent with the conclusions of other experiments. The specific-heat isotope effect and the normal-state susceptibility investigations<sup>15,16</sup>, the pressure and temperature dependence of the nuclear spin lattice relaxation<sup>17</sup>, and the muon spin relaxation ( $\mu\text{SR}$ ) experiments<sup>7</sup>, are also strongly indicative that the relevant excitation for the pairing mechanism is related to high-frequency intramolecular phonon modes, thus being consistent with a BCS weak-coupling limit.

Although tunnelling measurements<sup>6</sup> show a BCS-like density of states in the superconducting phase, they indicate a reduced gap ratio ( $2\Delta/k_B T_c$ ) larger than 5, implying a strong-coupling limit. On the other hand, a recent  $\mu\text{SR}$  experiment shows a reduced Hebel-Slichter peak, compared to the BCS prediction<sup>7</sup>. This latter result might indicate a significant broadening in the density of states  $D_s(E)$  in the superconducting phase. However, it remains to be seen whether this discrepancy of the gap ratio could be traced to such a broadening of  $D_s(E)$ .

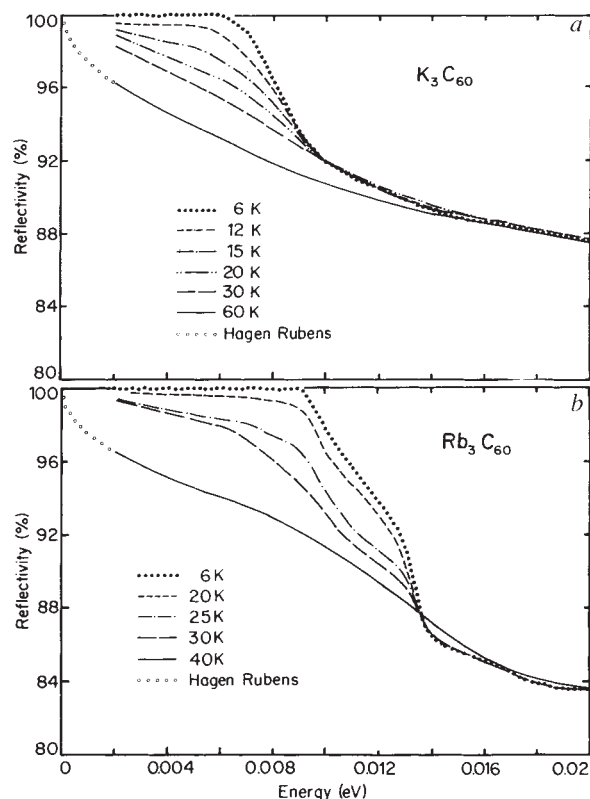


FIG. 2 Far-infrared reflectivity spectra above and below  $T_c$  of  $\text{K}_3\text{C}_{60}$  (a) and  $\text{Rb}_3\text{C}_{60}$  (b).

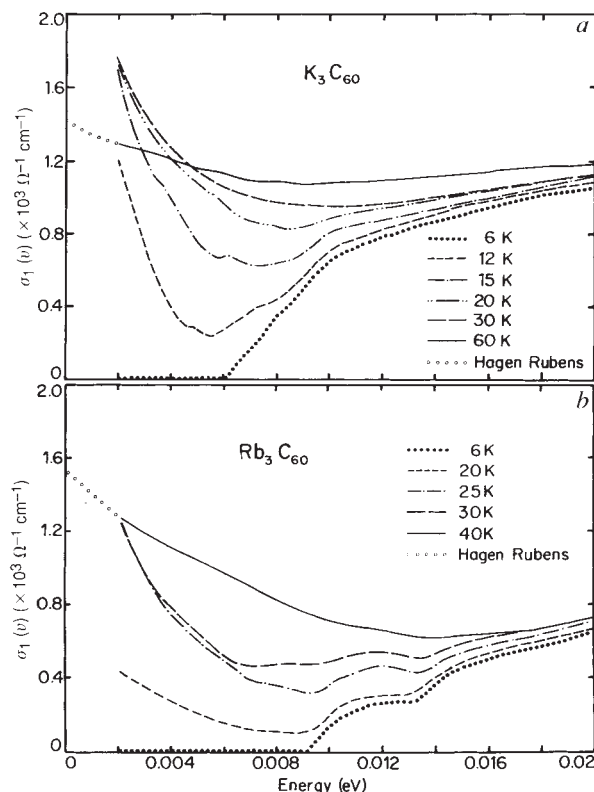


FIG. 3 Optical conductivity in the far-infrared above and below  $T_c$  of  $\text{K}_3\text{C}_{60}$  (a) and  $\text{Rb}_3\text{C}_{60}$  (b).



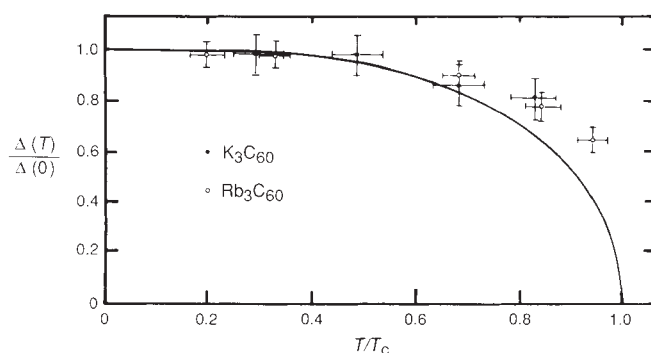


FIG. 4 Temperature dependence of the experimentally obtained superconducting gap (renormalized to  $\Delta(0) = 1.76k_B T_c$ ), compared with the weak-coupling limit of the BCS theory (solid line). The empty and filled circles refer to  $\text{Rb}_3\text{C}_{60}$  and  $\text{K}_3\text{C}_{60}$ , respectively. The error bars correspond to  $\pm 1$  K and  $\pm 0.25$  meV.

Figure 3 also displays the temperature dependence of the optical conductivity from the superconducting ground state up to the normal state. First, we note that the  $\text{K}_3\text{C}_{60}$  data (Fig. 3a) might be also suggestive of the appearance of the so-called coherence peak at low frequencies (below 3 meV). But because this feature is at the very lower end of our experimentally accessible frequency range, at present we consider this possibility very unlikely. The possibility needs to be investigated, however, by further experiments in the frequency range below the far-infrared. Nevertheless, the overall optical conductivity increases with increasing temperature due to the progressively larger amount of free charge carriers excited across the superconducting gap. The temperature dependence of the superconducting gap can be evaluated either by the maximum of the so-called relative reflectivity given by the ratio  $R_s/R_n$  of the reflectivity in the superconducting state over the reflectivity in the normal state, or by fitting the temperature dependence of the experimental ratio  $\sigma_{1s}/\sigma_{1n}$  with the Mattis–Bardeen BCS calculation<sup>14</sup>. Figure 4 shows the temperature dependence of  $\Delta$ , normalized to  $\Delta(0) = 1.76k_B T_c$ , as evaluated from the Mattis–Bardeen approach. While the general trend seems to be suggestive of a BCS-like behaviour, the gap ratio is always enhanced with respect to the BCS prediction as  $T_c$  is approached. Previous optical investigations<sup>8,18,19</sup> also indicated an anomalous temperature independence of  $\Delta$ . Thermal broadening, particularly close to  $T_c$  where a realistic determination of  $\Delta$  is impossible, might be important so that the intrinsic temperature dependence of  $\Delta$  is beyond the precision of the optical measurement<sup>8</sup>. Moreover, a gap distribution, due to a different  $T_c$  (that is, inhomogeneity of the specimens)<sup>18,19</sup> has been also suggested. Such a gap distribution can be, furthermore, the consequence of a gap anisotropy at the Fermi level, so that as the temperature is lowered below the onset of the transition, the regions with large gaps become superconducting first, with regions of smaller gaps following at lower temperatures<sup>18,19</sup>. Alternatively, the temperature dependence of the gap (Fig. 4) might be reminiscent of a fairly broad density of states, as also suggested experimentally by the  $\mu\text{SR}$  investigation<sup>7</sup>. All these effects cause the apparent weak temperature dependence of the gap feature when the temperature is raised towards  $T_c$ . □

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## Zeolite-encapsulated Mn(II) complexes as catalysts for selective alkene oxidation

Peter-Paul Knops-Gerrits, Dirk De Vos, Frédéric Thibault-Starzyk & Pierre A. Jacobs\*

Centrum voor Oppervlaktechemie en Katalyse, KU Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium

COMPLEXES of manganese(II) with bipyridine (bpy) have the potential to act as catalysts for oxidation of alkanes and alkenes when the complex is oxidized in acidic conditions<sup>1–6</sup>. But their catalytic activity in solution is limited by their catalase activity<sup>7</sup>—their tendency to decompose  $\text{H}_2\text{O}_2$ . Because of their polynuclear nature such complexes cannot induce epoxidation of alkenes, and other epoxidation catalysts suffer from self-oxidation and side reactions<sup>8–11</sup>. Moreover, all of these homogeneous catalytic processes require phase-transfer conditions. Here we report that, when encapsulated in the supercages of zeolites X and Y, *cis*-[Mn(bpy)<sub>2</sub>]<sup>2+</sup> complexes can catalyse selective epoxidation of alkenes without complications from competing processes such as self-oxidation or catalase activity. Epoxidation of cycloalkenes is followed by acid-catalysed ring-opening, carbon–carbon bond cleavage and formation of alkenedioic acids (Fig. 1). All of the various intermediates in the process can be obtained selectively by controlling the reaction conditions and zeolite acidity. Thus this supramolecular system provides a clean, one-step heterogeneous catalytic route to useful industrial products.

Synthesis of *cis*-manganese(II) bis-2,2'-bipyridyl complex encaged in zeolite NaX and NaY ([cMn(bpy)<sub>2</sub>]<sup>2+</sup>-X and [cMn(bpy)<sub>2</sub>]<sup>2+</sup>-Y), is performed by a stepwise method based on the synthesis of Fe and Ru tris-bipyridyl-Y complex<sup>12,13</sup>, but using the appropriate transition-metal to ligand ratio. MnY (or MnX) zeolite was prepared by ion exchange of NaY (or NaX) with a 2 mM aqueous solution of Mn(CH<sub>3</sub>COO)<sub>2</sub>. The MnY (MnX) zeolite containing 8 Mn<sup>2+</sup> ions per unit cell (1 Mn<sup>2+</sup> per supercage) was then washed, filtered and dried at 423 K under a N<sub>2</sub> flow. This material is used as reference in the catalytic experiments. In an inert atmosphere glove-box, 0.4 g of the bpy ligand was added to 2.0 g of the dried MnY (or MnX). The mixture was heated at 363 K for 24 h in a closed system to stimulate complex formation, then Soxhlet-extracted for 24 h with CH<sub>2</sub>Cl<sub>2</sub> to remove the unreacted ligand.

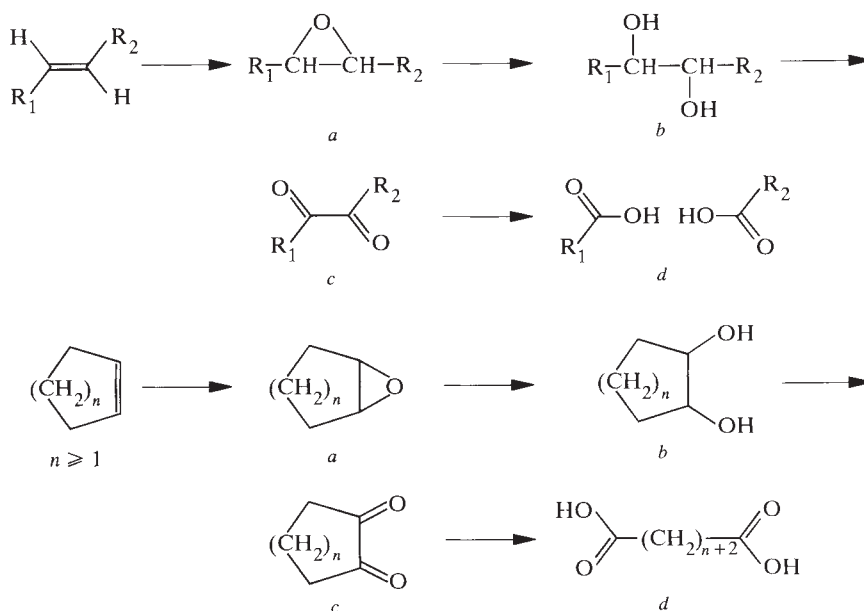
Thermo-gravimetric and chemical analysis (CA) performed on [cMn(bpy)<sub>2</sub>]<sup>2+</sup>-Y showed that after extraction an average number of  $2.02 \pm 0.05$  bpy per supercage and per Mn<sup>2+</sup> ion remain in the sample. This ratio corresponds to the proposed stoichiometry of the complex. Chemical analysis and electron spectroscopy for chemical analysis (ESCA) show that bulk- and

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\* To whom correspondence should be addressed.

FIG. 1 Scheme of the new catalytic chemistry achievable with  $cis\text{-}[\text{Mn}(\text{bpy})_2]^{2+}\text{-Y}$ : a, epoxide; b, diol; c, dione, and d, monomolecular  $\alpha$ ,  $\omega$ -alkanedicarboxylic acid or bimolecular  $\alpha$ -alk-anecarboxylic acid formation. New routes are opened to produce industrially relevant chemical intermediates, *inter alia* glutaric, adipic, suberic and sebacic acids, as well as dodecanedioic acid out of cyclopentene ( $n=1$ ), cyclohexene ( $n=2$ ), cyclooctene ( $n=4$ ), cyclododecene ( $n=6$ ) and cyclododecene ( $n=8$ ), respectively. The 1,2-diols produced on  $[c\text{Mn}(\text{bpy})_2]^{2+}\text{-Y}$  can be further oxidized: dione can be obtained from cyclohexene, and  $\alpha$ -hydroxy- $\beta$ -keto-alkane from 1-alkenes; carbonyl compounds from C-C bond cleavage (adipic acid from cyclohexene and benzaldehyde from styrene).



surface-Mn concentrations are equal, as the ratios of Mn to Si of 0.0494 (ESCA) and 0.0597 (CA), and Mn to Al of 0.1989 (ESCA) and 0.1379 (CA) are in good agreement, taking into account the known surface enrichment of silicon in the lattice<sup>14</sup>. The absence of complexes at the surface was confirmed by scanning electron microscopy. Fourier transform infrared spectra of  $[c\text{Mn}(\text{bpy})_2]^{2+}\text{-Y}$  show only minor frequency shifts from those of the homogeneous  $[c\text{Mn}(\text{bpy})_2]^{2+}$  complex<sup>1</sup>. The spectra of homogeneous *trans*- and *cis*-bipyridyl differ in the out-of-plane C-H deformation bands at  $765\text{ cm}^{-1}$ , which are split for the latter into two bands at  $757$  and  $772\text{ cm}^{-1}$  (refs 1, 15, 16) because of the lowering of the complex symmetry from  $D_{2h}$  to  $C_2$ . For  $[\text{Mn}(\text{bpy})_2]^{2+}\text{-Y}$  we see a broad band at  $772\text{ cm}^{-1}$ , characteristic of the *cis* complex. In homogeneous conditions, oxygen-containing ligands seem to direct to *cis*-coordination. Lattice oxygens and/or residual hydration water in the Mn(II) zeolite are thought to account for *cis*-complex formation; the vicinal position of the oxygen atoms may also be relevant. *Cis*-complex formation is furthermore enhanced by heating, by analogy with *trans*- $[\text{Ru}(\text{bpy})_2]^{3+}$  complexes, for which thermal *trans* to *cis* conversion is observed<sup>17</sup>. Electron paramagnetic resonance and diffuse reflectance spectroscopy (DRS) confirm the predominant  $2+$  state of Mn. Reflectance spectra of the pink  $[\text{Mn}(\text{bpy})_2]^{2+}\text{-Y}$  and  $-\text{X}$  samples show the characteristic metal-to-ligand charge-transfer transitions at  $495$  and  $530\text{ nm}$ , and the ligand-to-metal charge-transfer as a shoulder at  $358\text{ nm}$ . The intensity of these former absorptions is higher in NaX. An

increased nucleophilic character can be expected for Mn in NaY. These results are analogous to those of the bpy complexes of a zeolite-occluded isoelectronic  $d^5$  ion such as Ru(III) (ref. 14). For  $[\text{Ru}(\text{bpy})_3]^{3+}$ , bis-substitution is known to alter the optical spectrum significantly, producing bands at  $375$  and  $551\text{ nm}$  (ref. 18).

Molecular modelling shows that no distortion of the zeolite supercage ( $1.3\text{-nm}$  diameter) is necessary for inclusion of a  $[c\text{Mn}(\text{bpy})_2]^{2+}$  complex ( $1.2\text{-nm}$  diameter) (Fig. 2b). Upon transformation (Fig. 2a) of lattice-coordinated Mn(II) ( $\text{O}_L$ )<sub>3</sub> (with Mn in a type II cation site; ref. 19) into the *fac*-Mn(II) ( $\text{O}_L$ )<sub>3</sub>(N<sub>bpy</sub>)<sub>3</sub> complex (in which three type 2 zeolite oxygens ( $\text{O}_L$ ) of a six-membered ring are present in a plane under, and three bipyridyl nitrogen (N<sub>bpy</sub>) in a plane above, the Mn ion), the replacement of  $\text{H}_2\text{O}$  molecules by bpy ligands weakens the coordination of  $\text{Mn}^{2+}$  to the zeolite lattice (energy was calculated using a completed version of the MM + force field—a completed version of Allinger's MM2 (ref. 20)). The *fac*  $\leftrightarrow$  *cis* equilibrium should be thought of as the replacement of lattice type-II oxygen ions by a stronger ligand.

The room-temperature catalytic data illustrate the catalytic potential of the  $[c\text{Mn}(\text{bpy})_2]^{2+}\text{-Y}$  and  $-\text{X}$  catalyst (Table 1). A variety of olefinic substrates can be oxidized at room temperature, with intermediate to high conversion and selectivity. The products obtained with high selectivity are the epoxide (Table entries 1, 6, 8), the 1,2-diol (entries 4, 7, 9), and the  $\alpha$ ,  $\omega$ -alkanedicarboxylic acid (entries 5, 10). The three product types

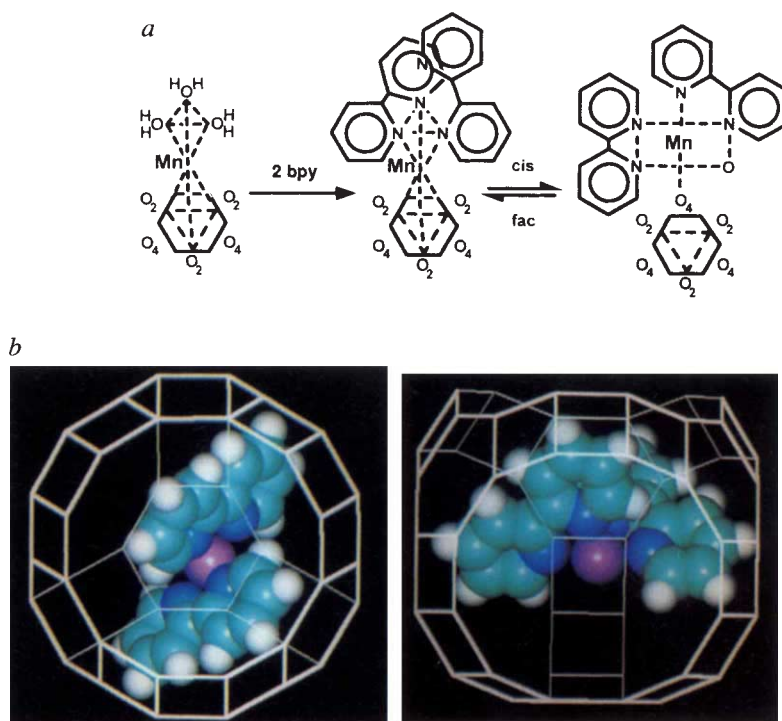
TABLE 1 Oxidation of alkenes on  $cis\text{-}[\text{Mn}(\text{bpy})_2]^{2+}$  in zeolites X and Y at  $293\text{ K}$

|    | Catalyst             | Substrate     | Conversion (%) | Selectivities (%) |      |        | Time (h) |
|----|----------------------|---------------|----------------|-------------------|------|--------|----------|
|    |                      |               |                | Oxide             | Diol | Diacid |          |
| 1  | Mnbpy <sub>2</sub> X | 1-hexene      | 22             | 81                | 14   |        | 4        |
| 2  | Mnbpy <sub>2</sub> Y | 1-hexene      | 20             | 50                | 40   |        | 18       |
| 3  | Mnbpy <sub>2</sub> X | Cyclohexene   | 41             | 62                | 32   |        | 4        |
| 4  | Mnbpy <sub>2</sub> Y | Cyclohexene   | 62             | 6                 | 79   |        | 18       |
| 5  | Mnbpy <sub>2</sub> Y | Cyclohexene   | 100            |                   |      | 80     | 40       |
| 6  | Mnbpy <sub>2</sub> X | 1-dodecene    | 11             | 74                | 22   |        | 4        |
| 7  | Mnbpy <sub>2</sub> Y | 1-dodecene    | 20             | 10                | 88   |        | 18       |
| 8  | Mnbpy <sub>2</sub> X | Cyclododecene | 38             | 78                | 22   |        | 4        |
| 9  | Mnbpy <sub>2</sub> Y | Cyclododecene | 56             | 4                 | 87   |        | 18       |
| 10 | Mnbpy <sub>2</sub> Y | Cyclododecene | 100            |                   |      | 84     | 40       |

Reactions were performed in a dynamic mode as follows: to  $0.2\text{ mmol}$  of complex and  $60\text{ mmol}$  of olefin substrate,  $30\%$  aqueous hydrogen peroxide was added at a rate of  $20\text{ mmol h}^{-1}$  until the desired  $\text{H}_2\text{O}_2/\text{substrate}$  ratio was obtained. In table entries 5 and 10, solvent volumes were increased to meet solubility requirements.



FIG. 2 *a*,  $cis-[Mn(bpy)_2]^{2+}$  complexes in zeolite Y: schematic representation of the  $cis-[Mn(II)(bpy)_2]$  complex formation. Lattice-coordinated hydrated  $Mn(II)$  first reacts with  $bpy$  to form a  $fac-[Mn(II)(O_L)_3(N_{bpy})_3]^{2+}$  intermediate and finally rearranges into a  $cis-[Mn(II)(bpy)_2(H_2O)_2]^{2+}$  complex with pseudo-octahedral coordination. (Note that the  $O_L$  present in a six-membered ring are either of type 2:  $O_2$  or type 4:  $O_4$ .) *b*, Space-filling molecular model (top and side view) (using the program Hyperchem from Autodesk Inc.) of  $cis-[Mn(bpy)_2]^{2+}-Y$  (structure derived from crystal data of non-encapsulated complex<sup>21</sup>). The edges and corners of the zeolite polyhedron represent O and Si(Al) atoms, respectively.



are formed consecutively, as can be seen from the selectivity changes at increasing substrate conversion (entries 3–5). The epoxide hydration is most probably acid catalysed, and the higher selectivity for the epoxide observed on  $MnX$  (entries 1, 2) might be related to the weaker acidity of this zeolite compared to  $MnY$ . Apart from this, both zeolites catalyse the same kind of chemical processes (entries 6, 7). Although Brönsted sites are not intentionally introduced in  $[cMn(bpy)_2]^{2+}-Y$  ( $Si/Al=2.4$ ) and are hardly measurable by conventional physico-chemical methods, the presence of small quantities stemming from  $Mn^{2+}$  hydrolysis and cation deficiencies cannot be excluded and seem sufficient to create a bifunctional catalyst (in zeolites the Brönsted-acid concentration increases and the strength decreases with decreasing  $Si/Al$  ratio). To isolate the pure epoxide, the acid strength can be modulated and the  $[cMn(bpy)_2]^{2+}-X$  ( $Si/Al=1.2$ ) catalyst minimizes epoxide opening. Once formed, epoxide can be further oxidized (Fig. 1), ultimately leading to (for example) adipic acid from cyclohexene and benzaldehyde from styrene. The deficiency in the selectivity balance is accounted for by allylic oxidation leading to small amounts of enol and enone. By changing the reaction time, the oxidant/substrate ratio and the nature of the solvent (or the temperature), sets of conditions can be selected in which each of the intermediate products (Fig. 1) can be obtained in high yields. If the peroxide is added slowly, the peroxide efficiency is significantly enhanced and the selectivities indicated can be obtained with almost stoichiometric peroxide/substrate ratios.

The catalyst remains effective for up to 1,000 cycles for cyclohexene oxidation with  $H_2O_2$ ; for example, with a peroxide/substrate ratio of 5. After drying of the catalyst at 320 K, the initial activity of the catalyst is obtained. Preliminary results showed that repeated catalyst regeneration is possible, indicating that through encapsulation of mono-nuclear complexes in a molecular sieve, their tendency to oxidative-destruction in reaction conditions is strongly suppressed. After repeated regeneration, changes in catalytic activity and spectroscopic properties of the complex (infrared, DRS) could not be detected.

The increase of electron density at the substrate double bond increases the reaction rate with  $[cMn(bpy)_2]^{2+}-Y$ , as observed with peracids and  $Mn$ -tetraphenylporphyrin (TPP) catalysed epoxidations<sup>9,10</sup>. Thus electronic rather than steric effects control the reaction. The catalase activity of uncomplexed manganese ions in zeolite Y always exceeds 95%, and is first order in manganese concentration. The low olefin conversion leads only to peroxide decomposition and formation of allylic oxidation products. The peroxide efficiency of the  $[cMn(bpy)_2]^{2+}-Y$  or  $-X$  catalysts seems therefore to be mainly determined by absence of uncomplexed  $Mn^{2+}$ . Typical peroxide selectivities obtained for cyclohexene oxidation are given in Table 2. When compared to recent data on titanium-substituted zeolite  $\beta$ <sup>22</sup> reaction, yields on peroxide basis are not inferior. When cyclohexene turnover rates are compared with Ti zeolites (including  $\beta$  as well as silicalite), significantly higher values are found for  $[cMn(bpy)_2]^{2+}-Y$ .

The reaction of *trans*- $\beta$ -methylstyrene and  $H_2O_2$  sheds light

TABLE 2 Comparison of cyclohexene oxidation with hydrogen peroxide at 298 K

| Catalyst*   | Catalyst (g) | Feed (mmol) | Substrate/ $H_2O_2$ | Time (h) | $H_2O_2$ conversion (%) | $H_2O_2$ selectivity (%) | Substrate conversion (%) | Turnover rate ( $h^{-1}$ ) |
|-------------|--------------|-------------|---------------------|----------|-------------------------|--------------------------|--------------------------|----------------------------|
| $Mnbpy_2Y$  | 0.4          | 60          | 0.33                | 4.0      | 51                      | 62                       | 9                        | 15                         |
| $Mnbpy_2Y$  | 0.4          | 60          | 1.00                | 4.0      | 37                      | 47                       | 17                       |                            |
| Ti- $\beta$ | 0.2          | 33          | 0.08                | 3.5      | 30                      | 83                       | 2                        | 4                          |
| Ti-silicate | 0.2          | 33          |                     | 3        |                         |                          |                          | 1                          |
| Ti- $\beta$ | 0.2          | 33          | 0.16                | 3.5      | 26                      | 73                       | 3                        |                            |

\*Abbreviations for catalysts as follows:  $Mnbpy_2Y$ ,  $cis-[Mn(bpy)_2]^{2+}-Y$  zeolite; Ti- $\beta$ , titanium-substituted zeolite  $\beta$  (ref. 22); Ti-silicalite, titanium-substituted silicalite (ref. 22).

on the epoxidation mechanism. In static conditions (0.02 mmol of catalyst, 2.5 mmol of olefinic substrate and 2.5 mmol of oxidant added together to 5 ml acetone at 293 K), a conversion of 31% (43 turnovers) with an epoxide selectivity of 39% (80% *trans* and 20% *cis*) is found. The high percentage of stereoretention in this case, and the relatively low amounts of allylic oxidation, rule out radical oxidation by the Haber–Weiss mechanism as the major mechanistic pathway. In epoxidations with Mn(porphyrin) and Mn(TPP), axially coordinating nitrogen bases increase the reaction rate, the selectivity in epoxide formation and the stereoselectivity<sup>9,10</sup>. A similar influence is probably exerted by the pyridyl that is *trans* to the oxygen in [cMn(bpy)]<sup>2+</sup>-Y. Hydrogen peroxide is activated by formation of a high-valent manganese species (for example, Mn(IV)=O) which forms a transition-state complex with the olefin. The oxene is probably inserted into the double bond of the olefin, which may or may not be coordinated itself to the Mn, thus giving different possible directional attacks. □

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SUPPLEMENTARY INFORMATION. Full details of the ESCA measurements, DRS and FTIR spectra (before and after catalytic operation) and SEM data are available on request. Requests should be addressed to Mary Sheehan at the London editorial office of *Nature*.

## Extreme and persistent drought in California and Patagonia during mediaeval time

Scott Stine

Department of Geography and Environmental Studies,  
California State University, Hayward, California 94542, USA

STUDIES from sites around the world<sup>1–5</sup> have provided evidence for anomalous climate conditions persisting for several hundred years before about AD 1300. Early workers emphasized the temperature increase that marked this period in the British Isles, coining the terms 'Mediaeval Warm Epoch' and 'Little Climatic Optimum', but many sites seem to have experienced equally important hydrological changes. Here I present a study of relict tree stumps rooted in present-day lakes, marshes and streams, which suggests that California's Sierra Nevada experienced extremely severe drought conditions for more than two centuries before AD ~ 1112 and for more than 140 years before AD ~ 1350. During these periods, runoff from the Sierra was significantly lower than during any of the persistent droughts that have occurred in the region over the past 140 years. I also present similar evidence from Patagonia of drought conditions coinciding with at least the first of these dry periods in California. I suggest that the droughts may have been caused by reorientation of the mid-latitude storm tracks, owing to a general contraction of the circumpolar vortices and/or a change in the position of the vortex waves. If this reorientation was caused by mediaeval warming, future natural or anthropogenically induced warming may cause a recurrence of the extreme drought conditions.

The Sierra Nevada is a high-elevation (to 4,400 m), 600-km-long fault block which trends northwest-southeast. Frontal passage between November and May accounts for >85% of the Sierran rain- and snowfall. Precipitation increases towards the east as the range crest is approached, at which point the average annual total approaches 2,000 mm. It decreases abruptly to the lee, averaging ~250 mm along the base of the eastern front.

Runoff at middle and high elevations is very seasonal, reaching a sharp peak during the months of greatest snowmelt (typically May–July). The Sierra is California's most important catchment area, providing two-thirds of the state's developed surface-water supply to its huge urban and agricultural systems.

Until recently, the most severe and persistent drought of California's instrumental record occurred between 1928 and 1934 (the 'Dust Bowl period'), when Sierran runoff averaged ~70% of normal. That interval was matched in severity during the 6 years 1987–1992, reinforcing the notion of a maximum 6- to 7-year dry spell. Evidence of mediaeval droughts that were of greater severity, and far greater duration, than either the Dust Bowl or the modern periods, appears at four sites in and adjacent to the central Sierra: Mono Lake, Tenaya Lake, the West Walker River and Osgood Swamp (see Table 1 for locations).

Mono Lake is a large (~18,000 ha) body of saline-alkaline water that abuts, and receives inflow from, the eastern front of the central Sierra. Because it lacks an outlet the lake fluctuates in response to climatic changes. Since 1940 the City of Los Angeles has diverted the influent streams for municipal supply, forcing a 14-m drop in lake level to an elevation of 1,942 m. But for diversions, today's 'natural level' would be ~1,957 m (ref. 6).

Two generations of relict stumps (of *Pinus jefferyi*, *Populus trichocarpa*, *Chrysothamnus nauseosus* and *Artemisia tridentata*) are rooted at low elevations on Mono Lake's artificially exposed shorelands. The oldest generation ('G-1 stumps') includes eight <sup>14</sup>C-dated individuals with basal elevations as low as 1,941.5 m (15.5 m below today's 'natural level', and slightly below the artificially depressed surface). Dates on outermost (death-year) wood from these eight are given in Table 1. When plotted with error bars (Fig. 1), the calibrated G-1 death-year dates overlap in a 96-year interval centred on AD 1112.

The second generation of Mono stumps ('G-2 stumps'), represented by five <sup>14</sup>C-dated individuals, stands rooted on the shorelands at elevations as low as 1,946 m (11 m below the 'natural level'). Calibrated <sup>14</sup>C dates on outermost rings from these five (Table 1), when plotted with error bars (Fig. 1), share a 46-year interval centred on AD 1350.

The G-1 and G-2 stumps provide evidence that Mono Lake fell to exceptionally low levels on two occasions during mediaeval time. The first of the lowstands, which persisted at least 50 years (the number of growth rings in the largest of the G-1



relicts), terminated around AD 1112 (the generalized death data on the G-1 stumps). The second lowstand, also at least 50 years in duration, terminated around AD 1350 (the generalized death data on the G-2 stumps). Independently dated lake-transgressive and lake-regressive sedimentary sequences from Mono Lake<sup>7</sup> (see Table 1 and Fig. 1, dates 1, 4, 6 and 16) confirm that the death of the G-1 and G-2 vegetation coincided with rises following lowstands, thus implicating drowning as the cause of death.

Because the Mono basin has been hydrographically stable (with no interbasin stream shifts) throughout Holocene time, and because tectonic warping, faulting, and lake-water displacement attributable to lake-bottom volcanism have not caused appreciable changes in lake level during the late Holocene<sup>8</sup>, it is reasonably concluded that on two occasions during mediaeval time low Sierran runoff drove Mono Lake to exceptionally low levels.

Tenaya Lake, 22 km west of the central Sierran crest, receives runoff from 2,100 ha of glaciated highlands (to elevation 3,335 m). This 90-ha water body is dammed by a moraine that has been breached by the effluent stream, forming a stable spillway at elevation 2,484 m. Stands of lodgepole pine (*P. contorta*) encircle Tenaya Lake down to its high-water line. Growth of trees below that line is precluded by annual inundation.

Protruding from Tenaya Lake are nine large, upright to moderately tilted lodgepole pine trunks. These snags, firmly anchored and apparently rooted, stand in 8–19 m of water. <sup>14</sup>C assays have been run on two of these trunks. Outermost wood from the oldest of the two provides a calibrated age range equivalent to AD ~1028–1159, contemporaneous with the G-1 Mono stumps (Table 1, Fig. 1). This relict stands in 13 m of water, and displays 68 annual rings at the water line (more rings undoubtedly exist at the presently-inundated tree base). Outermost wood from the younger of the two assayed trunks yields an age range of AD 1281–1386, similar to Mono's G-2 stumps (Table 1, Fig. 1). It stands in 11 m of water, and contains 141 annual rings at water line. The presence of these drowned trunks indicates that Tenaya Lake stood more than 13 m below its spillway for over 70 years before AD ~1093, and more than 11 m below it for over 141 years before AD ~1333. Because the Tenaya basin is hydrographically stable, and because there has been no geomorphic alteration of the spillway, low inflow seems the likely cause of the lowstands.

The West Walker River originates high on the east-Sierran flank. It debouches from its east-facing canyon at an elevation of 2,015 m, then turns abruptly northward and enters a deep, narrow-bottomed gorge. Dozens of Jeffrey pine stumps stand rooted on the lowest portions of the gorge floor, drowned by the stream. Radiocarbon assays on outermost wood from five of these reveal two generations of tree growth (Table 1). The

oldest, represented by an individual with ~220 annual rings, died within the interval AD 1044–1213, contemporaneous with the first of the inferred tree-killing transgressions at Mono and Tenaya lakes. The younger generation of West Walker River stumps, including individuals with as many as 140 annual rings, produces death-dates (Table 1) whose error bars (Fig. 1) exhibit a 94-year overlap centred on AD ~1339, equivalent to the G-2 dates at Mono and Tenaya lakes.

Jeffrey pine cannot survive complete basal inundation for periods exceeding several weeks during the season of active growth (A. Leiser, personal communication), which includes the period of peak runoff in the Sierra. The presence of the drowned stumps thus indicates that for periods of >200 years during mediaeval time, stream flows remained low enough to permit widespread tree establishment and growth on the narrow gorge bottom. Because the river at, and above, the stump site is hydrographically stable, and because the narrowness of the gorge floor over much of the stump-studded reach prevents horizontal stream shifts that would allow trees to colonize newly abandoned surfaces, neither stream piracy nor lateral migration can explain the presence of the stumps. As at Mono and Tenaya lakes, low Sierran runoff provides the most plausible explanation.

Osgood Swamp (elevation 1,995 m) is a small (5.6-ha) marsh that occupies a shallow, moraine-dammed depression immediately east of the Sierran crest. Inflow is limited to seepage from the surrounding ~100 ha. Mature lodgepole pines presently surround the marsh, but they are prevented from invading by perennially saturated ground.

In 1965, a rooted stump from the marsh was collected (D. P. Adam, personal communication) that exhibited 100 annual rings. Outside wood from this specimen provides a calibrated age range (Table 1) of AD 1024–1228, widely overlapping the death-year dates on the G-1 stumps at the West Walker River and at Mono and Tenaya lakes (Fig. 1). With no apparent evidence of late Holocene geomorphic or hydrographic alteration of Osgood Swamp, the >100 years of desiccation that permitted the establishment of trees is most reasonably attributed to low Sierran runoff.

Epic drought is not only the most reasonable explanation for the existence of stumps at each one of the presently aquatic sites described above, but it is the only plausible explanation for the site-to-site contemporaneity of the stumps. The two mediaeval droughts must have been more severe than the dry spell of the past 6 years. Throughout that 6-year period Osgood Swamp remained sodden, preventing colonization by trees; in each of those years the West Walker River experienced spring- and summertime flows too high and too prolonged to permit Jeffrey pines to establish or grow on its bed; Tenaya Lake overflowed for at least several weeks during the peak snowmelt period in

FIG. 1 Dates of drought terminations. The encircled numbers refer to the identity of the data source, as given in the first column of Table 1. The error bars represent  $1\sigma$ , calculated using the program CALIB 3.0.3. (ref. 11).

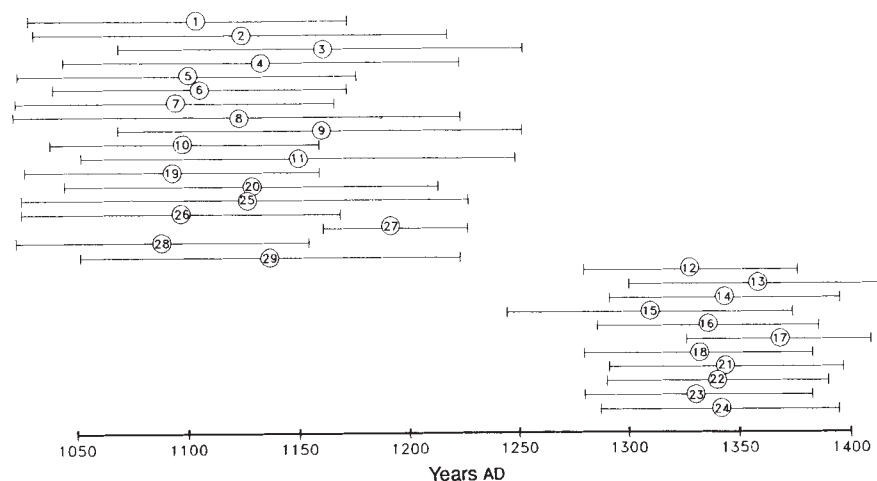


TABLE 1 Radiocarbon dates of drought terminations

Mono Lake, California (38° N, 110° W)

|                                                                                               | ID no.        | Lab.   | Date (yr BP) | Calibrated date*<br>(cal yr BP) | Sample description                           | Sample location                      | Significance                             |
|-----------------------------------------------------------------------------------------------|---------------|--------|--------------|---------------------------------|----------------------------------------------|--------------------------------------|------------------------------------------|
| 1.                                                                                            | USGS-1704†    | Menlo  | 930 ± 40‡    | 919–782<br>(AD 1931–1168)       | Pine cone in buried littoral sand            | E. wall Rush Cr., ele. ~1,944–47 m   | Dates rise from low stand of ~838 cal BP |
| 2.                                                                                            | USGS-1275†    | Menlo  | 910 ± 60     | 918–732<br>(AD 1032–1218)       | Tree stump rooted on Lee Vining Cr. delta    | E. of LV Cr., ele. 1,949.1 m         | Dates rise from low stand of ~838 cal BP |
| 3.                                                                                            | USGS-1276†    | Menlo  | 860 ± 60     | 886–695<br>(AD 1064–1255)       | Tree stump rooted on Lee Vining Cr. delta    | E. of LV Cr., ele. 1,949.7 m         | Dates rise from low stand of ~838 cal BP |
| 4.                                                                                            | USGS-1310†    | Menlo  | 890 ± 60     | 909–724<br>(AD 1041–1226)       | Pine cone in organic-rich lake-transgr. seds | E. wall LV Cr., ele. 1,949.1 m       | Dates rise from low stand of ~838 cal BP |
| 5.                                                                                            | USGS-1319†    | Menlo  | 940 ± 60     | 928–773<br>(AD 1022–1177)       | Shrub stump rooted on Mono shorelands        | ~600 m w. of LV Cr., ele. 1,942.1 m  | Dates rise from low stand of ~838 cal BP |
| 6.                                                                                            | USGS-1321†    | Menlo  | 920 ± 35     | 912–780<br>(AD 1038–1170)       | Grass rooted in buried littoral sands        | E. wall Rush Cr., ele. 1,944.5 m     | Dates rise from low stand of ~838 cal BP |
| 7.                                                                                            | USGS-1487†    | Menlo  | 950 ± 50     | 928–785<br>(AD 1022–1165)       | Tree stump rooted in Lee Vining Cr.          | E. side of LV Cr., ele. 1,951.2 m    | Dates rise from low stand of ~838 cal BP |
| 8.                                                                                            | UCLA-118†     | UCLA   | 920 ± 90§    | 931–724<br>(AD 1019–1226)       | Tree stump rooted on Lee Vining Cr. delta    | E. of LV Cr., ele. 'about 1,950 m'   | Dates rise from low stand of ~838 cal BP |
| 9.                                                                                            | LDGO-1677 FW† | Lamont | 860 ± 60     | 886–695<br>(AD 1064–1225)       | Tree stump rooted on Post Office Cr. delta   | W. of PO Cr., ele. 1,947.6 m         | Dates rise from low stand of ~838 cal BP |
| 10.                                                                                           | LDGO-1677 LW† | Lamont | 940 ± 20     | 914–790<br>(AD 1036–1160)       | Shrub stump rooted on Lee Vining Cr. delta   | E. wall of LV Cr., ele. ~1,947.3 m   | Dates rise from low stand of ~838 cal BP |
| 11.                                                                                           | CAMS-2506     | Liverm | 870 ± 60     | 898–702<br>(AD 1052–1248)       | Shrub stump rooted on 'Little Tahiti'        | Negit Islets, ML, ele. ~1,946 m      | Dates rise from low stand of ~838 cal BP |
| 12.                                                                                           | USGS-1277†    | Menlo  | 700 ± 60     | 671–571<br>(AD 1279–1379)       | Tree stump rooted on Lee Vining Cr. delta    | E. of LV Cr., ele. ~1,951.2 m        | Dates rise from low stand of ~600 cal BP |
| 13.                                                                                           | USGS-1315†    | Menlo  | 600 ± 70     | 650–534<br>(AD 1300–1416)       | Non-rooted pine stmp Lee Vining Cr. delta    | ~300 m w. of LV Cr., ele. ~1,942.4 m | Dates rise from low stand of ~600 cal BP |
| 14.                                                                                           | USGS-1167†    | Menlo  | 640 ± 60     | 658–550<br>(AD 1292–1400)       | Tree stump rooted on Lee Vining Cr. delta    | E. of LV Cr., ele. ~1,947.0 m        | Dates rise from low stand of ~600 cal BP |
| 15.                                                                                           | CAMS-2505     | Liverm | 730 ± 80     | 710–577<br>(AD 1240–1373)       | Shrub stump rooted on "Little Norway"        | Negit Islets, ML, ele. ~1,946 m      | Dates rise from low stand of ~600 cal BP |
| 16.                                                                                           | USGS-1316†    | Menlo  | 675 ± 50     | 663–561<br>(AD 1287–1389)       | Pine cones in buried littoral sands          | E. wall of LV Cr., ele. ~1,947.9 m   | Dates rise from low stand of ~600 cal BP |
| 17.                                                                                           | LDGO-1677 IW† | Lamont | 570 ± 30     | 623–539<br>(AD 1327–1411)       | Tree stump rooted on Lee Vining Cr. delta    | ~300 m e of LV Cr., ele. 1,950 m     | Dates rise from low stand of ~600 cal BP |
| Tenaya Lake, California (37° 50' N, 119° 27' W)                                               |               |        |              |                                 |                                              |                                      |                                          |
| 18.                                                                                           | Beta-46709    | BetaAn | 690 ± 60     | 669–564<br>(AD 1281–1386)       | Tree trunk rooted in Tenaya Lake             | Tenaya Lake, in 11 m of water        | Dates rise from low stand of ~600 cal BP |
| 19.                                                                                           | LDGO-1719 A   | Lamont | 950 ± 30     | 922–791<br>(AD 1028–1159)       | Tree trunk rooted in Tenaya Lake             | Tenaya Lake, in 13 m of water        | Dates rise from low stand of ~838 cal BP |
| West Walker River, California (Upstream of 'Chris Flat'; 38° 23' N, 119° 27' W)               |               |        |              |                                 |                                              |                                      |                                          |
| 20.                                                                                           | LDGO-1747 W   | Lamont | 904 ± 40     | 906–737<br>(AD 1044–1213)       | Tree stump rooted in W. Walker River         | W. Walker River near Chris Flat      | Dates rewatering of WWR, ~838 cal BP     |
| 21.                                                                                           | Beta-46675    | BetaAn | 640 ± 60     | 658–550<br>(AD 1292–1400)       | Tree stump rooted in W. Walker River         | W. Walker River near Chris Flat      | Dates rewatering of WWR, ~600 cal BP     |
| 22.                                                                                           | Beta-46676    | BetaAn | 660 ± 50     | 660–557<br>(AD 1290–1393)       | Tree stump rooted in W. Walker River         | W. Walker River near Chris Flat      | Dates rewatering of WWR, ~600 cal BP     |
| 23.                                                                                           | Beta-46681    | BetaAn | 690 ± 60     | 669–564<br>(AD 1281–1386)       | Tree stump rooted in W. Walker River         | W. Walker River near Chris Flat      | Dates rewatering of WWR, ~600 cal BP     |
| 24.                                                                                           | CAMS-2510     | Liverm | 660 ± 70     | 664–552<br>(AD 1286–1398)       | Tree stump rooted in W. Walker River         | W. Walker River near Chris Flat      | Dates rewatering of WWR, ~600 cal BP     |
| Osgood Swamp, California (38° 51' N, 120° 28' W)                                              |               |        |              |                                 |                                              |                                      |                                          |
| 25.                                                                                           | I-6843        |        | 910 ± 85     | 926–722<br>(AD 1024–1228)       | Tree stump rooted in Osgood Swamp            | Osgood Swamp                         | Dates rehydration of OS, ~838 cal BP     |
| Argentine Patagonia (Lago Cardiel, 48° 57' S, 71° 26' W; Catalon Marsh, 50° 28' S, 72° 58' W) |               |        |              |                                 |                                              |                                      |                                          |
| 26.                                                                                           | LDGO-1714 N   | Lamont | 945 ± 50     | 926–783<br>(AD 1024–1167)       | Organic detritus in lake-transgr. seds.      | Rio Cardiel delta, Lago Cardiel      | Dates rise from low stand of ~838 cal BP |
| 27.                                                                                           | LDGO-1714 H   | Lamont | 864 ± 40     | 787–722<br>(AD 1163–1228)       | Shrub stump rooted in buried soil            | Rio Tunas delta, Lago Cardiel        | Dates rise from low stand of ~838 cal BP |
| 28.                                                                                           | LDGO-1714 I   | Lamont | 972 ± 30     | 929–797<br>(AD 1021–1153)       | Organic detritus in lake-transgr. seds.      | Rio Tunas delta, Lago Cardiel        | Dates rise from low stand of ~838 cal BP |
| 29.                                                                                           | LDGO-1747 A   | Lamont | 880 ± 50     | 899–724<br>(AD 1051–1226)       | Tree stump rooted in marsh                   | Catalon marsh Lago Argentino         | Dates rehydrn. of marsh ~838 cal BP      |

\*Calculated at 1 $\sigma$  using CALIB 3.0.3. (ref. 3). Work is currently underway to establish dendrochronologically the precise timing of vegetation establishment and death. Abbreviations: PO, Post Office; ML, Mono Lake; Cr., creek; E., east; W., west; ele., elevation.

† Published previously, with less precise calibration, in ref. 3.

‡ Collected by M. Perkins in 1982 (personal communication).

§ Collected by D. B. Lawrence in 1960 (ref. 12).

|| Published previously, with less precise calibration, in ref. 5.



each of the six drought years, and at no time during those years did it fall more than 1 m below its sill. Because these three drainage basins are dominated by exposed bedrock, and contain little groundwater storage capacity, just a single dry year, rather than a sequence of dry years, is needed to cause low runoff. It is not just a lack of duration, therefore, but rather a lack of both severity and duration, that makes the modern dry spell an inappropriate analog for mediaeval drought.

Ring counts on the longest-lived of the G-1 and G-2 stumps indicate that the first of California's mediaeval droughts lasted for more than 220 years before the generalized termination date of AD 1112 (thus, from before AD ~892 to around AD 1112), whereas the second persisted for at least 141 years before AD 1350 (thus, from before AD 1209 to around AD 1350). The period of increased wetness that separated the two droughts persisted for less than 100 years (from AD ~1112 to some time before AD 1209). These decades were, even by historical standards, very wet, forcing Mono Lake to rise to 1961 m—a level higher than any attained in the past 150 years, and the second-highest lake stand of the last two millennia<sup>7</sup>. The mediaeval period in California was thus marked not only by severe and prolonged drought, but by abrupt and extreme hydroclimatic shifts—from inordinate dryness, to inordinate wetness, and back to dryness.

Similar evidence of wetland desiccation has recently been discovered in southernmost Patagonia (locations, Table 1), in the austral belt of westerly winds. Rooted in presently existing marshes and ponds near Bahia Catalon on Lago Argentino are stumps of southern beech (*Nothofagus sp.*) that display between 50 and 100 growth rings. Radiocarbon dating (Table 1) places the date of tree death in the range AD 1051–1226. Lake Cardiel, 200 km farther north, recovered from one of its lowest stands of the Holocene at this same time, drowning shrubs rooted at low elevations on its shorelands. Outermost wood from three of the drowned stumps produce death-dates (Table 1) that fall within the interval AD ~1021–1228<sup>9</sup>. All four of these death dates from Patagonian vegetation lie remarkably close to the drought-termination dates derived from California's G-1 stumps (Fig. 1).

The findings presented here support the notion that the mediaeval climatic anomaly was a global phenomenon. They indicate that during much of mediaeval time the planetary ocean-atmosphere system operated in a mode unlike that of modern time. The mid-latitude storm track of the northern hemisphere probably remained to the north of California during the mediaeval droughts, either because of a contraction of the circumpolar vortex (as occurred, to a moderate degree, during the drought of 1928–1934), or because of a persistent ridge of high pressure that steered cyclonic disturbances to relatively high latitudes (as occurred during the shorter drought of 1976–1977) (J. Monteverdi, personal communication). The former condition is consistent with the position of the westerlies across mid-North America during the centuries before AD 1150, as reconstructed by Baerrels and Bryson<sup>2</sup>. Vortex contraction could also account for the south Patagonian drought, though for different reasons. Because the Patagonian sites are at a higher latitude than those in California (48°–50° S, as opposed to 38°–39° N), and because they are not only leeward of, but also hydrographically isolated from, the Andes, a vortex contraction could place the zone of strongest westerly flow directly over the region, intensifying the Andean rain-shadow, and causing the inferred desiccation.

The aberrant atmospheric circulation of mediaeval time seems to have brought to some regions of the world a far greater departure in precipitation than in temperature. With this in mind, and to avoid prejudicing future palaeoclimatic analyses, reference to a 'Mediaeval Warm Period' or a 'Little Optimum', except when applied locally, should be replaced with some other phrase (for example, 'Neo-Atlantic'<sup>10</sup> or 'Mediaeval Climatic Anomaly') that avoids a precise characterization of conditions.

California's mediaeval precipitation regime, if it recurred with today's burgeoning human population, would be highly disruptive

environmentally and economically. This emphasizes the importance of considering changes in precipitation, rather than simply in temperature, when weighing the potential impacts of future global climate change. □

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## Rapid cycling of high-molecular-weight dissolved organic matter in the ocean

Rainer M. W. Amon & Ronald Benner

Marine Science Institute, University of Texas at Austin, Port Aransas, Texas 78373-1267, USA

**DISSOLVED organic matter (DOM) in the ocean is one of the largest active reservoirs of organic carbon on Earth. It is important to understand the processes by which DOM is recycled, particularly as changes in the oceanic DOM pool could affect atmospheric carbon dioxide concentrations on timescales of 1,000 to 10,000 years (ref. 1). It is commonly believed that low-molecular-weight material, which comprises 65–80% of DOM<sup>2–5</sup>, is rapidly remineralized, and that high-molecular-weight material is refractory. But the average age of DOM in the deep ocean is about 6,000 years (ref. 6) which implies that a large proportion of the DOM cycles only very slowly. Here we present a study of the relative bioavailability of low- and high-molecular weight DOM in water samples taken from the northern Gulf of Mexico during a diatom bloom. Bacterial growth and respiration in the presence of high-molecular-weight DOM were respectively three and six times greater than for low-molecular-weight material. Although both of these pools undoubtedly contain mixtures of compounds with varying reactivities and turnover times, our results demonstrate that the bulk of oceanic DOM comprises small molecules that cycle slowly and are relatively unavailable to microorganisms.**

The bioreactivity of oceanic DOM was investigated in May 1992 during a cruise in the northern Gulf of Mexico. Surface water was collected on the Louisiana shelf (18° 54.9' N, 89° 55.5' W) during a diatom bloom. The water sample was filtered through a 0.1-µm-pore filter to remove particulate material, and the DOM in the filtrate was separated into high-molecular-weight (HMW) and low-molecular-weight (LMW) fractions by tangential-flow ultrafiltration (~1 nm pore size, corresponding to  $M_r$  ~1,000)<sup>2</sup>. The HMW fraction included all colloidal particles of size <0.1 µm. The initial concentration of dissolved organic carbon (DOC) was 152 µM, and ~30% of the DOC was HMW. A natural bacterial assemblage from the same water was added to the HMW and LMW fractions to

investigate the rates of bacterial growth and remineralization supported by the two size classes of DOM. Duplicate water samples were incubated in glass bottles in the dark at ambient temperatures (22–25 °C).

Rates of carbon consumption were examined by measuring changes in dissolved oxygen and DOC concentrations. Dissolved oxygen concentrations were determined by Winkler titration using an autotitrator<sup>7,8</sup>. DOC concentrations were measured by high-temperature combustion<sup>9</sup> after filtration of water samples through a pre-combusted glass-fibre filter. Most bacteria passed the filter and were included in the DOC measurement. These two independent measures of carbon consumption were in general agreement (Fig. 1a, b; Table 1), indicating that DOC consumption was due to bacterial remineralization rather than particle formation or sorption on the container walls.

Dissolved oxygen and DOC concentrations in HMW incubations decreased rapidly, and dropped well below concentrations in LMW incubations (Fig. 1a, b). Oxygen consumption in HMW incubations was high and continuous throughout the 132-h incubations, indicating that most of the HMW DOM was rapidly mineralized to carbon dioxide by bacteria. In contrast, oxygen consumption was low in LMW-DOM incubations and appeared to cease after 38 h, indicating that only a small fraction of LMW DOM was readily available for bacterial utilization. Respiration rates in HMW incubations ( $1.23 \mu\text{M O}_2 \text{ h}^{-1}$ ) were 6 times higher than in LMW incubations ( $0.19 \mu\text{M O}_2 \text{ h}^{-1}$ ; Table 1). The rates in HMW incubations are similar to, but lower than, respiration rates ( $1.99 \mu\text{M O}_2 \text{ h}^{-1}$ ) measured at the same location in unfractionated surface water (J. D. Pakulski *et al.*, manuscript in preparation), suggesting that sample handling and manipulations had minimal effects on microbial remineralization processes. Lower respiration rates are more typical for these waters during the absence of phytoplankton blooms<sup>10</sup>. Results from additional experiments during non-bloom periods also indicate that HMW DOM cycles faster than LMW DOM, but rates of bacterial remineralization were 2–3 times lower during non-bloom conditions. (R.M.W.A. and R.B., manuscript in preparation).

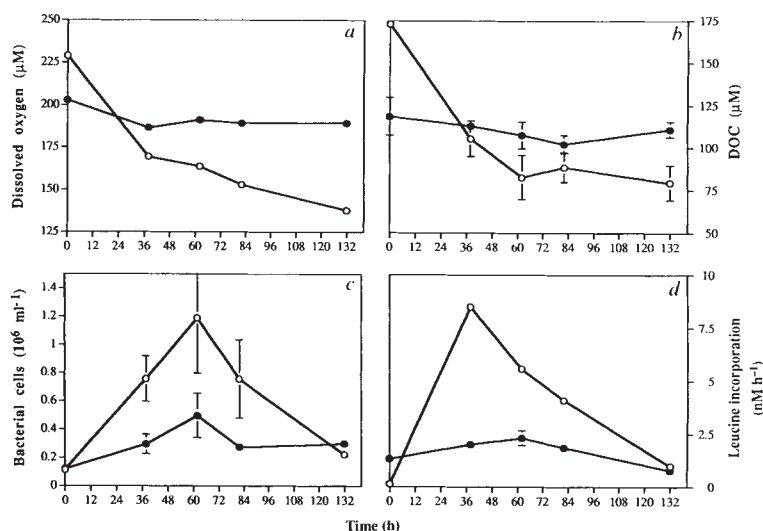
Bacterial abundances<sup>11</sup> increased linearly during the first 62 h of the incubations, and reached peak values of  $1.19 \times 10^6$  cells per ml in HMW incubations and  $4.97 \times 10^5$  cells per ml in LMW incubations (Fig. 1c). HMW DOM clearly supported greater cell

production than LMW DOM. This was also shown in humic and clearwater lakes<sup>12</sup>, where LMW supported lower bacterial yields than HMW DOM. Bacterial abundances decreased after 62 h due to grazing by protozoans or viral lysis, so all rates in Table 1 were calculated for the initial 62 h incubation period. The incorporation of [<sup>3</sup>H]-leucine was measured as an indicator of bacterial protein synthesis and growth<sup>13</sup>. Leucine incorporation was greater in HMW incubations (Fig. 1d), further indicating that HMW DOM supported relatively high rates of bacterial growth. Rates of bacterial production with HMW DOM ( $0.40 \mu\text{M C h}^{-1}$ ) were nearly 3 times higher than rates with LMW DOM (Table 1).

Nutrient dynamics were dramatically different between HMW and LMW incubations. There was net uptake of inorganic nitrogen ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) in HMW incubations ( $0.13 \mu\text{M N h}^{-1}$ ) and net nitrogen regeneration in LMW incubations ( $0.03 \mu\text{M N h}^{-1}$ ; Table 1). These differences in nitrogen dynamics indicate that bioreactive constituents of HMW and LMW DOM are compositionally distinct. Based on rates of bacterial carbon production in HMW incubations (Table 1) and assuming a C/N ratio of 4 for bacteria<sup>14</sup>, we estimate a bacterial nitrogen demand of  $0.1 \mu\text{M N h}^{-1}$ . This nitrogen demand is similar to the measured net uptake of inorganic nitrogen indicating that inorganic nitrogen, rather than organic nitrogen, served as the primary nitrogen source for bacterial growth on HMW DOM. In LMW incubations, however, there was net regeneration of inorganic nitrogen indicating that bioreactive LMW DOM was relatively rich in organic nitrogen; this is consistent with the observed relationship between nitrogen regeneration and substrate C/N ratios<sup>15</sup>. It appears that bioreactive HMW DOM has a high C/N ratio, which is consistent with a predominantly polysaccharide composition<sup>2</sup>. Bioreactive LMW DOM has a relatively low C/N ratio, which is consistent with a predominantly amino-acid composition.

The uptake of inorganic nitrogen by bacteria during the utilization of DOM in a phytoplankton bloom has been observed previously<sup>16</sup>, and may be characteristic of bacterial growth on phytoplankton-derived HMW DOM. The availability of nitrogen could regulate bacterial utilization of HMW DOM and the temporal and spatial coupling between its production and consumption. The low concentrations of bioavailable nitrogen

FIG. 1. Bacterial oxygen consumption (a), bacterial consumption of dissolved organic carbon (DOC) (b), bacterial abundance (c), and bacterial leucine incorporation (d) in seawater batch cultures with natural DOM of different molecular weights. Open circles represent sea water with high-molecular-weight (HMW) DOM and solid circles represent sea water with low-molecular-weight (LMW) DOM. Each point represents the average of two replicate experiments, and the error bars represent their mean deviation. Surface water (27 practical salinity units) was filtered through a Nuclepore filter cartridge (0.6- $\mu\text{m}$  pore size) to remove particles and bacteriophages. The filtrate was passed through a hollow-fibre ultrafilter (0.1- $\mu\text{m}$  pore size) to concentrate bacterial cells<sup>31</sup> for later inoculation of the batch cultures. DOM in the filtrate was separated into HMW and LMW fractions using a polysulphone ultrafilter with a cutoff of  $M_r$  1,000 (ref. 2). The concentrate (2 l) was diluted with artificial sea water to a similar initial DOC concentration as the LMW incubation. Sea water with the different size classes of DOM was distributed into acid-rinsed bottles (2.5 l), inoculated with bacterial cells, and nitrate and phosphate were added to give concentrations 2–3 times higher than ambient. The bottles were sealed without a headspace and subsampled through Teflon tubing and valves<sup>10</sup>. Bacterial abundance was determined by epifluorescence microscopy using diaminophenylindole as a stain<sup>11</sup>. Bacterial leucine incorporation was determined according to Kirchman *et al.*<sup>13</sup>. Triplicate samples (10 ml) and one killed control were incubated



for 0.5 h with 10 nM final concentration of [<sup>3</sup>H]-leucine (specific activity 60 Ci mmol<sup>-1</sup>).



TABLE 1. Bacterial utilization of dissolved organic matter

| Sample type | Respiration rate ( $\mu\text{M O}_2 \text{ h}^{-1}$ ) | DOC consumption rate ( $\mu\text{M C h}^{-1}$ ) | Bacterial Production ( $\mu\text{M C h}^{-1}$ ) | Nutrient dynamics ( $\mu\text{M N h}^{-1}$ ) |                 | Growth efficiency (%) | Fraction of DOC consumed (%) | DOC turnover time (d) |
|-------------|-------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|----------------------------------------------|-----------------|-----------------------|------------------------------|-----------------------|
|             |                                                       |                                                 |                                                 | $\text{NO}_3^-$                              | $\text{NH}_4^+$ |                       |                              |                       |
| HMW         | 1.23                                                  | 1.46                                            | 0.40                                            | -0.10                                        | -0.03           | 25                    | 52                           | 6                     |
| LMW         | 0.19                                                  | 0.18*                                           | 0.14                                            | +0.02                                        | +0.01           | 42                    | 9                            | 26                    |

The dissolved organic matter (DOM) was collected from the northern Gulf of Mexico during a diatom bloom. The sample was separated into high-molecular-weight (HMW:  $M_r > 1,000$ ) and low-molecular-weight (LMW:  $M_r < 1,000$ ) fractions by ultrafiltration. Abbreviations: DOC, dissolved organic carbon; - indicates nitrogen uptake; + indicates nitrogen regeneration. All rates were calculated using data from the initial and 62 h time points. Bacterial production was calculated from the leucine incorporation rate using the empirical conversion factor  $4.46 \times 10^{16}$  cells per mol-Leu (ref. 10) and a cell-to-carbon conversion value of 20 fg-C per cell (ref. 14). Growth efficiency was calculated from bacterial respiration and production, assuming a respiratory quotient of 1.  $[\text{NO}_3^-]$  and  $[\text{NH}_4^+]$  were measured on an Alpkem nutrient autoanalyser<sup>30</sup>. DOC turnover times were calculated by dividing the initial DOC concentration by the corresponding respiration rate. Carbon normalized rates of respiration, DOC consumption, and bacterial production were  $0.59 \mu\text{M O}_2 \text{ h}^{-1}$  per mg DOC,  $0.70 \mu\text{M C h}^{-1}$  per mg DOC and  $0.19 \mu\text{M C h}^{-1}$  per mg DOC, respectively, for HMW DOM, and  $0.13 \mu\text{M O}_2 \text{ h}^{-1}$  per mg DOC,  $0.13^* \mu\text{M C h}^{-1}$  per mg DOC and  $0.10 \mu\text{M C h}^{-1}$  per mg DOC, respectively, for LMW DOM.

\* Analysis of variance indicated that the slope of a linear regression of the data was not significantly different from zero ( $P = 0.324$ ).

typically present in oceanic surface waters could delay bacterial remineralization of HMW DOM until it mixes below the euphotic zone where nutrients are in higher concentration. The production of HMW DOM and its delayed remineralization could explain the recently observed overconsumption of inorganic carbon relative to nitrogen during spring phytoplankton blooms<sup>17,18</sup>.

Differences in the compositions of bioreactive HMW- and LMW-DOM appear to be reflected in bacterial growth efficiencies on these substrates. Bacterial growth efficiencies were higher (42%) with LMW DOM than with HMW DOM (25%; Table 1). Nitrate was the major nitrogen source for bacterial growth with HMW DOM (Table 1), and this could result in relatively low bacterial growth efficiencies because of the high energy demand associated with nitrate assimilation. In contrast, organic nitrogen supported the relatively high bacterial growth efficiency on LMW DOM. Independent estimates of bacterial growth efficiencies on LMW- and HMW-DOM were calculated using changes in cell abundance (Fig. 1c) and an estimate of 20 fg C per cell (ref. 14). The same relative trend in growth efficiencies was observed, with an estimated growth efficiency of 2% with HMW DOM and 9% with LMW DOM. These conservative values are similar to those estimated previously (2–9%) for bacterial growth on DOM during a phytoplankton bloom<sup>16</sup>. The above independent estimates of growth efficiency differed greatly depending on the measured parameters (leucine incorporation versus abundance). We believe that cell yield data underestimate bacterial growth because of cell losses due to protozoan grazing and viral lysis during the incubation. Therefore, growth efficiency estimates based on leucine incorporation are presented in Table 1.

During the initial 62 h of incubation, 52% ( $\sim 90 \mu\text{M C}$ ) of HMW DOM was consumed whereas only 9% ( $\sim 11 \mu\text{M C}$ ) of LMW DOM was consumed (Table 1). Carbon turnover times ranged from 6 d for HMW DOM, to 26 d for LMW DOM (Table 1). Turnover times are presented to emphasize the large relative difference in bioreactivity of the two size classes of DOM rather than to provide true estimates of cycling times. Both HMW- and LMW-DOM pools undoubtedly contain mixtures of compounds of varying reactivities and turnover times. It is apparent, however, that LMW DOM contains a much larger fraction of refractory components with longer inherent residence times in the ocean.

Past investigations<sup>2,5</sup> indicate that 20–35% of oceanic DOM is of HMW ( $M_r > 1,000$ ). Chemical characterizations of HMW DOM isolated from sea water by tangential-flow ultrafiltration revealed that material from surface waters is dominated by polysaccharides, whereas unsubstituted alkyl and aromatic structures comprise a greater fraction of HMW DOM from deep waters<sup>2</sup>. Based on the abundance and depth distribution of HMW DOM<sup>2</sup>

and polysaccharides<sup>2,19,21</sup> it was speculated that they were important components of the upper-ocean carbon cycle. Similarly, depth distributions of colloid abundance<sup>22</sup> and measurements of  $^{234}\text{Th}$  activity in colloids<sup>23</sup>, indicate short residence times for these components of HMW DOM. Our results are consistent with a short residence time for colloids, but they indicate that bacterial remineralization rather than particle aggregation is the major fate of this size class of DOM. Patterns of nutrient utilization during bacterial degradation of HMW DOM further indicated that these components are depleted in organic nitrogen and therefore likely to be polysaccharides. Similar dynamics were shown in a continuous culture experiment with sea water where carbohydrates supported most bacterial carbon and energy demand, and inorganic nitrogen supported most bacterial nitrogen demand<sup>24</sup>.

The realization that most LMW DOM in sea water is very resistant to bacterial degradation is unexpected and intriguing. About 65–75% of DOM in surface waters is LMW ( $M_r < 1,000$ )<sup>2–5</sup> and as much as 80% of the DOM in the deep sea is LMW<sup>2</sup>. Thus, the bulk of DOM in the ocean is small molecules that cycle slowly and are relatively unavailable to microorganisms. This conclusion is consistent with the old apparent radiocarbon age of DOC in the ocean<sup>6</sup>. Attempts to characterize LMW DOM have been largely unsuccessful<sup>25</sup>. Hama and Handa<sup>26</sup> found that biomolecules, such as carbohydrates, proteins and organic acids, accounted for  $\sim 70\%$  of HMW DOM but only  $\sim 2\%$  of LMW DOM in lake water. There are several possible explanations for the low bioreactivity and lack of chemical identification of LMW DOM, including the occurrence of unusual biomolecules that are selectively preserved<sup>27</sup>, the shielding of biomolecules by physical and chemical protection mechanisms<sup>28</sup>, and the presence of unusual structures produced during non-enzymatic oxidation and reduction reactions<sup>29</sup>. The characterization of LMW DOM and the mechanisms of its production and destruction should lead to a better understanding of the ocean carbon cycle. □

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## Formation of the Muzo hydrothermal emerald deposit in Colombia

T. L. Ottaway\*, F. J. Wicks\*, L. T. Bryndzia†, T. K. Kyser‡ & E. T. C. Spooner§

\* Department of Mineralogy, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6, Canada  
 † 345 West Gaywood Drive, Houston, Texas 77079, USA

‡ Department of Geological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

§ Department of Geology, University of Toronto, 22 Russell Street, Toronto, Ontario, M5S 3B1, Canada

FOR over 1,000 years, the emerald deposits of Colombia have been the principal source of the world's largest and finest gem-quality emeralds—a variety of beryl containing chromium and vanadium<sup>1</sup>. Whereas most emerald deposits are found in association with igneous host rocks<sup>1</sup>, the Colombian deposits occur in organic-rich black shales, and their origin in the absence of any evidence of igneous activity has been a persistent enigma. Here we present evidence from the Muzo mine (located about 100 km from Bogotá) that hydrothermal brines transported evaporitic sulphate to structurally favourable sites, where it was thermochemically reduced. We suggest that the sulphur generated by this process reacted with organic matter in the shales to release trapped chromium, vanadium and beryllium, which in turn enabled emerald formation.

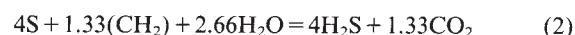
Earlier studies have established that emerald mineralization on the eastern and western flanks of the Cordillera Oriental of Colombia is structurally related to tectonic blocks formed by the intersection of regional fault zones striking north-northeast and northwest. The tectonic blocks that contain emerald mineralization have also been metasomatically altered by Na and Ca<sup>2,3</sup>. Our study focused on the Muzo mine, one of the largest emerald deposits in Colombia, located ~100 km north-northwest of Bogotá within one of these tectonic blocks on the western flank of the Cordillera Oriental<sup>4</sup>. Mineralization occurs within a single horizon which lies within a thick sequence of Lower Cretaceous, organic-rich shales and limestones of the Villeta Formation<sup>5</sup>. The organic matter has been pyrolysed to impsonite (a partially crystalline, carbon-rich variety of pyrobitumen) during deep burial. Vitrinite was not observed. The emer-

ald mineralization occurs in cavities (vugs) within a network of thin (<8 cm), calcite–albite–pyrite veins (Fig. 1) which are stratabound and fracture controlled. These veins are often severely brecciated and contain clasts of host rock. Calcite is the most abundant vein mineral, although locally it may be absent, and accessory quartz and barite may be abundant. Fluorite (CaF<sub>2</sub>) and parisite ((Ce, La)<sub>2</sub>Ca(CO<sub>3</sub>)<sub>3</sub>F<sub>2</sub>) occur as accessory minerals, and are successfully used as indicators of emerald mineralization. Pyrite is ubiquitous throughout the veins and the host shales, with some concentration at the vein–wallrock contact.

We have found that the emerald-bearing veins forming the Muzo deposit are spatially related to highly altered, irregular zones of host shales, which are called *cenicero* (Spanish for 'ash'). These *cenicero* zones are depleted in organic matter, are grey-white in colour and contrast strongly with the surrounding unaltered, organic-rich, black shales. They are brecciated and contain fragments of calcite, albite, muscovite and pyrite crystals, and doubly terminated quartz crystals in a matrix of carbonate and native sulphur. Emeralds occur in the narrow distal portions of the calcite–albite–pyrite veins that radiate from the *cenicero* zones, but rarely occur within the *cenicero*. Trapiche emeralds, six-rayed emerald crystals intergrown with shale, occur sporadically in the shale immediately surrounding the *cenicero*. These sulphur-bearing zones are quite distinctive from, and should not be confused with, the bright-yellow encrustations of rocks in dormant mining properties. The latter is the supergene alteration of pyritic shales; in contrast, the *cenicero* zones are grey-white in colour, with unweathered, pristine pyrite crystals in veins extending from these sites, and trapiche emeralds around their perimeter.

Our measurements show that the fluid inclusions in Muzo emeralds are NaCl saturated (40 wt% NaCl ± KCl) and contain CO<sub>2</sub> (liquid and vapour), H<sub>2</sub>O and a mixture of gases (CH<sub>4</sub> and N<sub>2</sub>). In addition, composite Ca–Fe chloride salts, Sn daughter minerals and Mg, Mn and Ti in the precipitates from decrepitated inclusions have been described<sup>6,7</sup>. Homogenization coincides with NaCl dissolution at 325 °C ± 10 °C (1σ; n = 154)<sup>8</sup>. This temperature represents a minimum trapping temperature, and the actual temperature of formation may be greater by ~50 °C if the deposits formed at pressures of ~1 kbar as suggested<sup>9</sup>. Differences between the δ<sup>18</sup>O values of quartz and calcite in the veins give temperatures of ~400 °C. The hypersalinity, an estimated δ<sup>18</sup>O value of +17‰ for the fluid inferred from the oxygen isotropic composition of quartz and calcite, the high formation temperature of quartz and emerald, and δD values near –60‰ from fluid inclusions in emeralds, suggest that the mineralizing solutions were residual brines from evaporites or, more likely, basinal fluids that had interacted with evaporites. Others have also proposed an evaporite fluid source<sup>6–10</sup>.

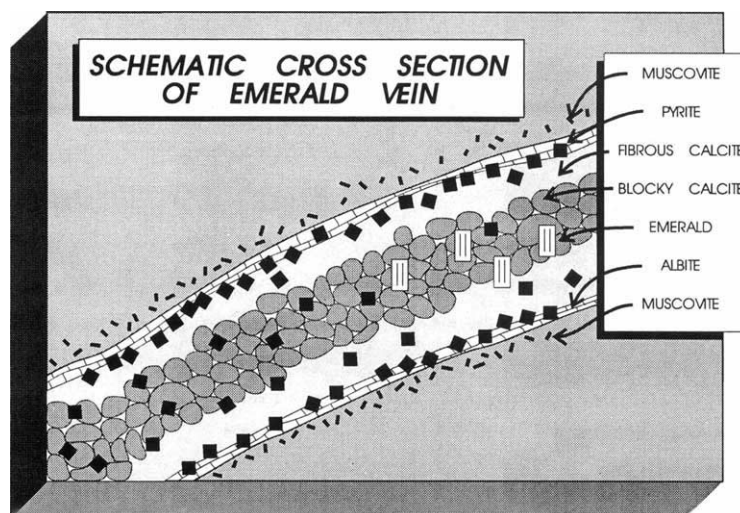
We propose that as the hydrothermal brines moved along faults and unconformities into structurally favourable sites, local concentrations of bitumen-derived H<sub>2</sub>S (δ<sup>34</sup>S, –3 to +6‰) thermochemically reduced the evaporite sulphate (δ<sup>34</sup>S ≈ +17‰) to produce native sulphur and pyrite with δ<sup>34</sup>S values varying between +8 to +17‰. The sulphur, in turn, may have reacted with more organic matter to produce additional H<sub>2</sub>S through the reactions:



These reactions have been studied by Orr<sup>11,12</sup> over the temperature range 175 to 250 °C. Both reactions are exothermic, so once initiated could have become self-sustaining until all of the organic matter within the shale at the reaction sites was consumed. The high reflectance values of pyrobitumen that we obtained from the shales immediately surrounding the *cenicero* zones ( $R_0$  = 6.5–8.0% compared with  $R_0$  = 2.3–4.5% for the shale in general) suggest that the *cenicero* zones, now ash-coloured



FIG. 1 Schematic cross-section of an emerald-bearing vein. Fine muscovite grains occur in the shale at the vein margins, with albite lining the veins at the contacts. The precipitation of albite was followed by tabular or fibrous calcite. Lastly, individual euhedral emerald crystals (or pockets of crystals) precipitated contemporaneously with, or were succeeded by, blocky anhedral calcite. The width of a typical vein is ~3–7 cm.



from the loss of organic matter and containing native sulphur, are the likely remnants of the reaction sites that produced the mineralizing hydrothermal brines.

The calcite in the emerald-bearing veins radiating from the *cenicero* zones is isotopically light,  $\delta^{13}\text{C} = -21\%$ , and indicates that organic matter was the probable source of  $\text{CO}_2$ . The measured  $\text{CO}_2$  densities of fluid inclusions in emeralds, using  $\text{CO}_2$  liquid-to-vapour homogenization temperatures, range from 0.02 to 0.25 g cm $^{-3}$  and indicate that the emeralds crystallized under a varying fluid pressure. We propose that the generation of  $\text{CO}_2$  from organic matter during the reduction of sulphate (reactions (1) and (2)) would have caused pressure to build in the reaction site. This could have resulted in the episodic explosive expulsion of fluids from the *cenicero* zones, fracturing the enclosing shales and brecciating the pre-existing vein material. This fluctuating pressure regime and pulses of fluid from the *cenicero* reaction zones into the emerald-bearing veins would explain the repeated fracturing and healing of emerald crystals by new, often deeper green, layers of emerald.

Recent studies of metalliferous black shales have found that organic matter can be a critical agent in the concentration, mobilization and precipitation of metals $^{13-16}$ . We have found that most of the organic matter has been consumed in the *cenicero* reaction zones; these zones are the locus of both the emerald-bearing veins, and the shale-hosted trapiche emerald crystals. Beus $^2$  found that Cr and V occurs with the carbonaceous portion of the host shales, and we suggest that Be also occurs in the organic matter.

The amount of Be required to produce the observed quantities of emerald is surprisingly small. At 300 °C and pH 4, using the average Be concentration of  $3 \pm 0.5$  p.p.m. (refs 2, 4) in the Muzo shales, a solution need only contain a  $\text{Be}^{2+}$  concentration of  $10^{-7}$  molal (moles per kg solution) to account for the quantity of emerald estimated to be present in a total vein space of 5,000 m $^3$  (ref. 17). Thus, there is more than enough Be present in the system to produce the observed quantities of emerald $^{7,8}$ . Most of the Be still remains locked in the shale organic matter, but in the *cenicero* where the organic compounds have undergone reaction, the Be was made available.

Three samples of the *cenicero* were analysed by direct-coupled-plasma emission spectrometry and showed a slight decrease in Be content to  $2.1 \pm 0.5$  p.p.m. which is just statistically significant. Similarly the principal colouring agent of emerald, Cr at  $29 \pm 5$  p.p.m., is lower in the *cenicero* than in the shales at  $38 \pm 5$  p.p.m., and V at  $46 \pm 5$  p.p.m. also is lower in the *cenicero* than the shales at  $83 \pm 5$  p.p.m. This decrease in Be, Cr and V in the *cenicero* is consistent with their release from the organic

matter in the black shales during sulphate reduction, and subsequent mobilization into the emerald forming hydrothermal brines.

This is consistent with our model which suggests that Be was only released into the hydrothermal brines when the host organic matter was oxidized by sulphate reduction. Furthermore, Be forms  $\text{OH}^-$  complexes in low-pH solutions $^{17}$ , hence the production of  $\text{H}_2\text{S}$  and  $\text{OH}^-$  in the *cenicero* (reactions (1) and (2)) would have created the ideal conditions for complexing and transporting this metal once it became available. According to this model, some of the Be was carried in the hydrothermal brines out from the *cenicero* zones under pressure into the veins,

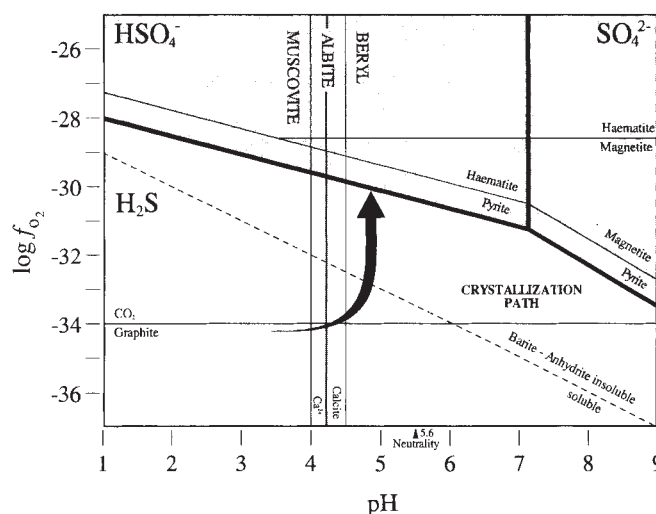


FIG. 2 A schematic pH–log  $f_{\text{O}_2}$  diagram ( $f_{\text{O}_2}$  is oxygen fugacity) for the Muzo mineral assemblage at 300 °C (ref. 3). The stability fields for graphite (representing organic matter), haematite, pyrite, barite, calcite, muscovite, albite and emerald were calculated using the following activities: S, 0.1; C, 1;  $\text{Ba}^{2+}$ ,  $10^{-3}$ ;  $\text{K}^+$ , 0.5;  $\text{Na}^+$ , 5.0;  $\text{Ca}^{2+}$ , 1.5;  $\text{Be}^{2+}$ ,  $10^{-9}$ ;  $\text{H}_2\text{O}$ , 0.75. The emerald veins lie within the pyrite, calcite and barite fields. The key reactions relating muscovite, albite and emerald and their calculated equilibrium constants are:  $3\text{Na}^+ + \text{muscovite} + 6\text{quartz} \rightarrow 3\text{albite} + \text{K}^+ + 2\text{H}^+$  (log  $K$  at 300 °C =  $-10.54$ );  $2\text{muscovite} + 12\text{quartz} + 9\text{Be}^{2+} + 6\text{H}_2\text{O} \rightarrow 3\text{beryl} + 2\text{K}^+ + 16\text{H}^+$  (log  $K$  at 300 °C =  $+11.06$ );  $2\text{albite} + 3\text{Be}^{2+} + 2\text{H}_2\text{O} \rightarrow \text{beryl} + 2\text{Na}^+ + 4\text{H}^+$  (log  $K$  at 300 °C =  $+10.71$ ). The general path of fluid evolution as deduced from the mineral assemblages is represented by the black arrow.

and some simply diffused out into the host rock where it crystallized, incorporating shale, to form the trapiche emeralds around the perimeter of the reaction sites.

The mineralized veins (Fig. 1) are characterized by a sequential precipitation of muscovite, albite, calcite and emerald which follows a path of increasing pH and oxygen fugacity (Fig. 2). We suggest that the precipitation of these minerals resulted from wallrock reactions with the H<sub>2</sub>S-rich hydrothermal brines. Initially, Fe in the shale would have reacted with H<sub>2</sub>S to precipitate pyrite. The acidic fluid produced by this reaction may have dissolved the carbonate in the wallrock to produce the increased pH in the vein solutions (see Fig. 2 caption).

The removal of Fe from the system as pyrite is a crucial factor in the development of the spectacular colour of Muzo emeralds<sup>18</sup>. Chromium and V not only impart a blue-green colour to beryl, but also a red fluorescence that intensifies the colour to produce a luminous quality<sup>19</sup>. This fluorescence is quenched by the presence of Fe<sup>3+</sup> in the beryl structure which may be why, in general, Fe-bearing emeralds from pegmatite-ultramafic environments do not have the same intensity of colour as their Colombian counterparts<sup>19</sup>.

The emeralds we have examined from the Cosquez mine north of Muzo and from the Chivor mine on the eastern flank of the Cordillera Oriental contain fluid inclusions remarkably similar to those in the Muzo emeralds. This, and the similar geological settings, strongly suggests that the thermochemical reduction of sulphate was a major factor in the development of all these deposits. Thus we predict that reaction sites, analogous to the *cenicero* zones at Muzo, may be found at other Colombian emerald deposits. Native sulphur may or may not be present depending on whether intermediate reaction products have been

preserved. It is interesting to note that an enormous pocket of emeralds, with an estimated value of several million dollars, was found in conjunction with a *cenicero*-like zone in December 1989 at the Chivor mine, on the eastern flank of the Cordillera Oriental (G. Jara, personal communication 1989). □

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## Bilateral hand representation in the postcentral somatosensory cortex

Yoshiaki Iwamura, Atsushi Iriki & Michio Tanaka

Department of Physiology, Toho University School of Medicine, 5-21-16 Omori-Nishi, Otaku, Tokyo 143, Japan

IN accordance with its important role in prehensile activity, a large cortical area is devoted to representation of the digits. Within this large cortical zone in the macaque somatosensory cortex, the complexity of neuronal receptive field characteristics increases from area 3b to areas 1 and 2 (refs 1–7). This increase in complexity continues into the upper bank of the intraparietal sulcus, where the somatosensory cortex adjoins the parietal association cortex. In this bank, callosal connections are much denser than in the more anterior part of this cortical zone<sup>8–17</sup>. We have now discovered a substantial number of neurons with receptive fields on the bilateral hands. It was previously thought that neuronal receptive fields were restricted to the contralateral side in this cortical zone. Neurons with bilateral receptive fields were not found after lesioning the postcentral gyrus in the contralateral hemisphere. The majority of these neurons had receptive fields of the most complex types, representing multiple digits, indicating that the interhemispheric transfer of information occurs at higher levels of the hierarchical processing in each hemisphere.

The responses of isolated single cells were recorded from four awake, well-behaved monkeys of both sexes, using techniques described elsewhere<sup>3,5,7</sup>. In one hemisphere, the entire digit representation of the postcentral gyrus was explored in a total of 50 penetrations and the responses of 863 units were studied in

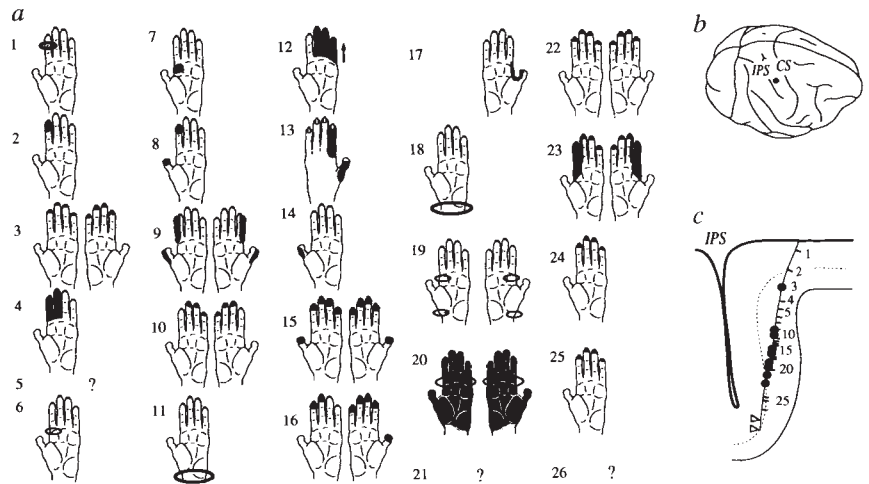
detail. All neurons with bilateral receptive fields were found in the upper bank of the intraparietal sulcus (Fig. 1). In the penetration depicted in Fig. 1, neurons at nine recording sites had bilateral receptive fields that covered either multiple digit tips, both the first and second digits, the dorsum of the hand, or the entire glabrous hand. The receptive fields on multiple digit tips constituted the majority in this and other penetrations (31 out of 72 neurons with bilateral hand receptive fields recorded in this hemisphere) and, in general, the receptive field size and orientation of the bilateral receptive fields were symmetrical.

The symmetry observed for the location and size of receptive fields also applies to the direction in which the stimulus was presented, namely those that responded to strokes in one direction but not in the opposite direction (Fig. 2). This was true for all of five neurons which were directionally selective. In the case shown in Fig. 2, the spatial configuration of the receptive fields in the two hands was the same, but the response to the left hand was much stronger. We found that among 105 neurons with bilateral receptive fields, 61 responded better to stimulation of the contralateral side (58.1%), 9 preferred ipsilateral stimulation (8.6%), and 35 responded about equally to stimulation of either side (33.3%); their response intensity was estimated by the maximal instantaneous firing rate in the peristimulus histogram, as shown in Fig. 2.

The distribution of cells with bilateral receptive fields is shown in Fig. 3. Sections A and B are taken from a relatively medial region passing across either the forearm or fifth digit representation; sections C–E are from the more lateral digit representation. Bilateral receptive fields were found in the upper part of the bank (area 2), but the majority were found in the middle part of the bank, across the cytoarchitecturally ambiguous border separating area 2 from areas 5 or 7 (refs 7, 18, 19), and were in both supra- and infragranular layers. Among 105 neurons with bilateral receptive fields, 72 had receptive fields



FIG. 1 a, Receptive fields (numbered 1–26) of neurons recorded along an electrode penetration in the upper bank of the intraparietal sulcus. The extents of skin receptive fields are shaded in black. Effective positions of joint manipulation neurons are circled. Neurons having no identifiable receptive fields are indicated by a question mark. The arrow at receptive field 12 indicates the preferred direction of a moving stimulus across the receptive field. A total of 9 neurons had bilateral receptive fields along this penetration. Their recording sites are indicated by filled circles in c. Note the symmetry in the ipsilateral and contralateral receptive field configuration in most of these pairs. b, A dot on the postcentral gyrus indicates the entry of the electrode penetration in the same animal from which receptive fields were determined in a. CS, central sulcus; IPS, intraparietal sulcus. c, A section perpendicular to CS that includes the electrode track shown in a. Sites of neuronal recordings are indicated by either horizontal bars (for unilateral receptive fields) or filled circles (for bilateral receptive fields). Numbers represent the locations of neurons corresponding to receptive fields



depicted in a. Open triangles at the end of the electrode track indicate sites of lesions used as landmarks for later reconstruction of recording sites. Dotted line indicates layer IV.

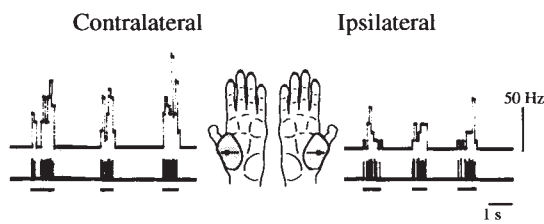


FIG. 2 Comparison of the maximal response of a neuron to stimulation of either hand. This neuron responded preferentially to a moving stimulus applied to its receptive fields (shaded) in a particular direction (preferred directions indicated by arrows). The response peak to skin strokes on the ipsilateral hand (right) was about a half of that of the contralateral hand (left). Top traces: discharge frequencies at every 0.1-s bin. Bottom traces: discriminated spike discharges. Horizontal bars below the traces indicate timing of strokes over the skin.

restricted to the hand. Bilateral receptive field of neurons in the medial region (sections A and B in Fig. 3) included, in addition to the multiple digit tip type, those that extended to the forearm from the hand or those that were restricted to the axial trunk on the body midline. No neurons had bilateral receptive fields confined to the proximal part of the forelimb.

These results were confirmed in three additional monkeys which served as controls in a study whose purpose was to learn whether ipsilateral responses are transmitted through the contralateral hemisphere. In one monkey, the digit region of the postcentral gyrus was completely ablated; in two other monkeys, multiple injections of ibotenic acid were made into several sites of the bilateral digit representation in the postcentral gyrus, after identifying the sites by multi-unit recording. After either procedure, no bilateral receptive fields were found in the postcentral gyrus of the contralateral side.

The density of callosal connections varies among different cortical cytoarchitectonic fields and among different somatosensory representations. In the digit region of the postcentral somatosensory cortex in macaque monkeys, callosal connections are nearly absent in area 3b, sparse in area 1, less sparse in area 2, and rather dense in the more caudal part of the cortex, areas 5 and 7 (refs 12, 13). On the other hand, the intraparietal zone receives intrinsic corticocortical projections from the more anter-

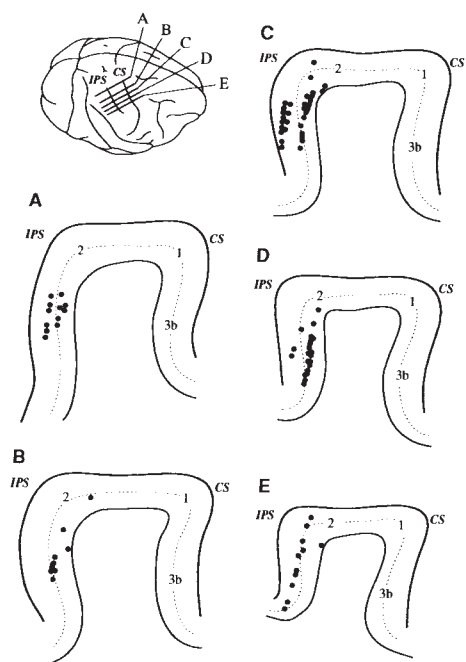


FIG. 3 Recording sites (dots) of neurons with bilateral receptive fields on five off-sagittal brain sections (A–E) orthogonal to the central sulcus of one hemisphere. Sections A and B are from a relatively medial region where neuronal receptive fields often extend to the forearm or the trunk; sections C–E are from the more lateral digit region. CS, central sulcus; IPS, intraparietal sulcus. Dotted lines indicate layer IV. Numbers 3b, 1, 2 and 5 indicate cytoarchitectonic subareas.

ior part of the postcentral gyrus<sup>20</sup>. Thus, the bilaterality of neurons, presumably conferred by callosal connections, occurs at the higher levels of hierarchical processing where integration of signals from the two hands can take place. Neurons that are activated by the use of either hand and neurons with receptive fields covering both arms have been reported in the more medial part of the postcentral gyrus, area 5 (refs 21–24), but not in the lateral cortical zone for digit representation, as studied here. Our findings confirm our previous observation that neurons can be driven by stimulation of either hand<sup>7</sup>.

The region of bilateral hand representation may be involved in integrating information necessary for the co-operative actions of the two hands, which includes the perception of objects explored by the two hands. It may also be the station for information transfer across the midline for tactile discrimination. Such transfer is lost after callosal sections<sup>25,26</sup>. Although it has been proposed that the second somatosensory area (SII) is involved in these functions<sup>16,17,27</sup>, our results indicate that the intraparietal zone may also participate in this information transfer. The intraparietal zone projects to SII and the surrounding areas<sup>28</sup> where neurons with bilateral receptive field are found<sup>29,30</sup>. Thus intraparietal regions may work in conjunction with the lateral parietal areas to integrate information from both hands. □

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## Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene

Hiroshi Takeshima<sup>\*†</sup>, Masamitsu Iino<sup>‡</sup>, Hiroaki Takekura<sup>§</sup>, Miyuki Nishi<sup>\*||</sup>, Junko Kuno<sup>||</sup>, Osamu Minowa<sup>||</sup>, Hiroshi Takano<sup>||</sup> & Tetsuo Noda<sup>||</sup>

<sup>\*</sup> International Institute for Advanced Studies, Shimazdu N-80,

1 Nishinokyo-Kuwahara-cho, Kyoto 604, Japan

<sup>‡</sup> Department of Pharmacology, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

<sup>§</sup> Department of Physiology, National Institute of Fitness and Sports, 1 Shiromizu, Kanoya, Kagoshima 891-23, Japan

<sup>||</sup> Department of Cell Biology, Cancer Institute, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan

CONTRACTION of skeletal muscle is triggered by the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum (SR) after depolarization of transverse tubules<sup>1,2</sup>. The ryanodine receptor exists as a 'foot' protein in the junctional gap between the sarcoplasmic reticulum and the transverse tubule in skeletal muscle, and is proposed to function as a calcium-release channel during excitation-contraction (E-C) coupling<sup>3–6</sup>. Previous complementary DNA-cloning studies have defined three distinct subtypes of the ryanodine receptor in mammalian tissues, namely skeletal muscle, cardiac and brain types<sup>7–12</sup>. We report here mice with a targeted mutation in the skeletal muscle ryanodine receptor gene. Mice homozygous for the mutation die perinatally with gross abnormalities of the skeletal muscle. The contractile response to electrical stimulation under

physiological conditions is totally abolished in the mutant muscle, although ryanodine receptors other than the skeletal-muscle type seem to exist because the response to caffeine is retained. Our results show that the skeletal muscle ryanodine receptor is essential for both muscular maturation and E-C coupling, and also imply that the function of the skeletal muscle ryanodine receptor during E-C coupling cannot be substituted by other subtypes of the receptor.

In the mouse skeletal muscle ryanodine receptor gene, exon 1, which codes for the first 17 amino acids of the molecule, and exon 2, which codes for the following 40 amino acids, were located by comparing the genomic DNA sequence with the cDNA sequence (Fig. 1a). To disrupt the ryanodine receptor gene, a replacement vector (pMSkRRS10) was constructed in which a neomycin-resistance gene was inserted into exon 2 and a viral thymidine kinase gene was attached to the genomic fragment. J1 ES cells<sup>13</sup> were transfected with the targeting vector and cultured in the presence of the drugs G418 and FIAU. Among 61 doubly resistant clones isolated, four separate clones, including numbers 136 and 160, were shown by Southern blot hybridization analysis to contain the mutated gene (Fig. 1b). This mutant allele will be referred to as *skrr*<sup>ml</sup> to distinguish it from other mutations, such as that responsible for malignant hyperthermia, at the same locus. Chimaeric male mice were generated by injecting the ES cells into blastocysts, and bred to yield mice heterozygous for the *skrr*<sup>ml</sup> allele.

Of 146 neonates obtained by crosses between mice heterozygous for *skrr*<sup>ml</sup>, 41 were wild type (+/+), 70 were heterozygous (+/*skrr*<sup>ml</sup>), and 35 were dead neonates with the homozygous genotype (*skrr*<sup>ml</sup>/*skrr*<sup>ml</sup>). This distribution is thought to follow the mendelian rule and indicates that the *skrr*<sup>ml</sup>/*skrr*<sup>ml</sup> mice survive fetal development. The *skrr*<sup>ml</sup> mutation is lethal perinatally in the homozygous state, probably due to respiratory failure because the *skrr*<sup>ml</sup>/*skrr*<sup>ml</sup> mice failed to breathe, remained light purple and did not move after birth. The mutant neonates displayed an abnormal curvature of the spine, thin limbs and a thick neck area, and the skeleton preparations showed an abnormal rib cage and an arched vertebral column (Fig. 1c, d). These abnormalities caused by the *skrr*<sup>ml</sup> mutation are similar to those of mice lacking myogenin<sup>14,15</sup> and muscular dysgenic (*mdg*) mice<sup>16,17</sup>. The *mdg* mutation is expressed in skeletal muscle as a failure of E-C coupling and the absence of the

<sup>†</sup> To whom correspondence should be addressed at: Department of Neurochemistry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156, Japan.



skeletal muscle dihydropyridine (DHP) receptor<sup>18-20</sup>. Therefore, the gross anatomical abnormalities common to these three lines of mutant mice may result from deficiency of functional skeletal muscle.

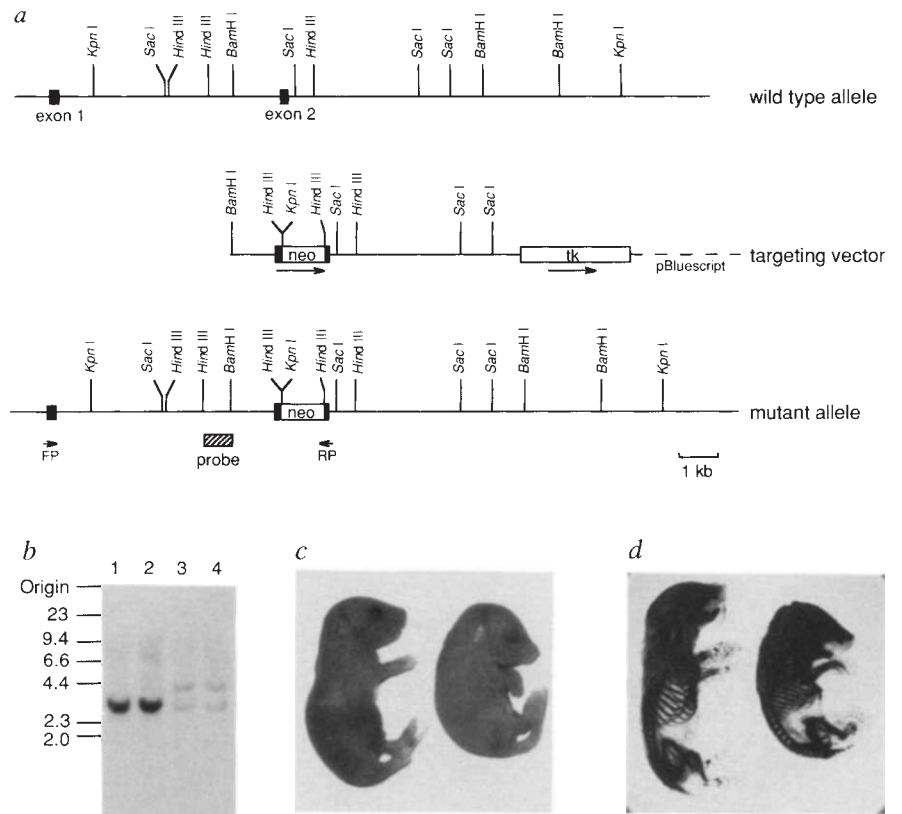
We next analysed RNA and protein products derived from the mutant gene (Fig. 2). RNA blot hybridization analysis with a probe specific for the skeletal muscle ryanodine receptor (Fig. 2a) showed that skeletal muscle from the *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> mice contains a hybridizable RNA species slightly larger than that from wild-type mice. The RNA species in the homozygous mutant was shown by reverse-transcribed polymerase chain reaction (RT-PCR) experiments (Fig. 2b) to carry the translation-termination codons and the sequence of the neomycin-resistance gene, introduced by the targeting vector, in its 5'-terminal region corresponding to exon 2. Immunoblotting analysis (Fig. 2c) indicated that the homozygous mutant muscle lacks the high-*M<sub>r</sub>* membrane protein recognized by polyclonal antibody against the rabbit skeletal muscle ryanodine receptor. Thus, it is likely that the *skrr*<sup>m1</sup> is a null mutation of the skeletal muscle ryanodine receptor.

**FIG. 1** Homologous recombination at the skeletal muscle ryanodine receptor locus (a, b) and physical features of *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> mice (c, d). **a**, Schematic representation of the targeted disruption. Restriction-enzyme maps of the 5'-terminal region of the mouse skeletal muscle ryanodine receptor gene, targeting vector (pMSkRRS10) and predicted mutant allele are shown. The first and second exons are indicated by filled boxes. The neomycin-resistance gene (*neo*) and the virus thymidine kinase gene (*tk*) are shown by open boxes; the transcriptional directions are indicated by arrows. **b**, Detection of homologous recombination by Southern blot analysis. DNA samples (30 µg each) digested with *Sac*I from embryonic stem (ES) clones: numbers 240 (lane 1), 234 (lane 2), 160 (lane 3) and 136 (lane 4) were analysed. The probe indicated in **a** detects ~3.3 kb and ~4.4 kb fragments, that are generated from the wild-type and targeted allele, respectively. Clones 240 and 234 were not identified as having been targeted. The size markers are indicated in kilobases (kb). **c**, Newborn mice with the +/*skrr*<sup>m1</sup> (left) or the *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> (right) genotype. **d**, Skeleton preparations<sup>25</sup> of neonates with the +/*skrr*<sup>m1</sup> (left) or the *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> (right) genotype. The +/*skrr*<sup>m1</sup> mice showed no apparent abnormalities in comparison with wild-type mice.

**METHODS.** A mouse skeletal muscle cDNA library was screened using a cDNA probe from pRR705<sup>7</sup>. Among the clones obtained, λMSkRR2 and -6 carry the 5'-noncoding and amino-terminal coding sequences for mouse ryanodine receptor mRNA. Then, a mouse genomic library was screened using a probe derived from λMSkRR2. Several clones including λMSkRRG6 and -8 were thus isolated, and partial sequence of the genomic DNA was determined<sup>26</sup> to locate exons 1 and 2 in the gene. Targeting vector (pMSkRRS10) was constructed from genomic fragments derived from λMSkRRG6, synthetic *Pst*I linkers, the 1.1-kb *Xho*I-*Bam*HI fragment from pMC1neo polyA (Stratagene), the ~2.8 kb *Xho*I-*Sac*I fragment containing the thymidine kinase cassette<sup>27</sup> and pBluescript SK(-) (Stratagene). In the vector, the neomycin-resistance gene attached with the synthetic linkers (GTGAATGAATGAAGCTTGGTACCTCGACGAATTACGTCACCTACTTACTTCGAACCATGGAGCTGCTTAAAGCT at the 5' end and GATCAATTCAAGCTTCTGCA TTAAGTTCGAAG at the 3' end), was inserted into the *Pst*I site in exon 2. Furthermore, the former linker introduced translation-termination codons (TGAATGAATGA). ES clone J1<sup>13</sup> were transfected with pMSkRRS10 linearized with *Bam*HI by electroporation and

The morphological features of skeletal muscle in *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> mice were examined (Fig. 3). Most of the muscle fibres from the +/*skrr*<sup>m1</sup> neonates were fully striated, filled with myofibrils, and had peripherally located nuclei (Fig. 3a, c). In contrast, skeletal muscle from the *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> neonates was characterized by degeneration. The fibres were small and fragmented by loose and amorphous tissues, and their nuclei still remained centrally located (Fig. 3b). A similar deficiency of muscle fibres was also discerned in dorsum muscle from homozygous mutants (not shown). Electron microscopy demonstrated that myofibrils in muscle fibres of *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> neonates were variable in size, and mostly smaller and fewer than in +/*skrr*<sup>m1</sup> neonates (Fig. 3d). However, longitudinal sections revealed no abnormalities in the structure of sarcomeres (Fig. 3e, f). These histological features of muscle from *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> mice are similar to those from *mdg* mice<sup>21</sup>.

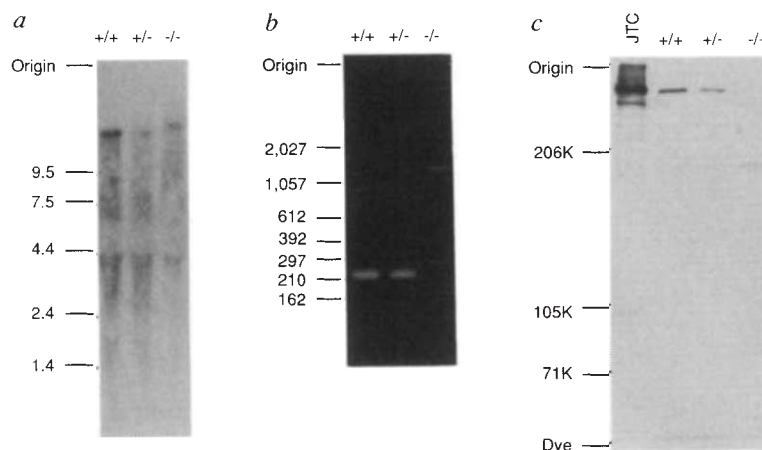
We then examined E-C coupling of skeletal muscle (Fig. 4). Electrical stimulation in physiological salt solution (PSS) induced twitch contractions in skeletal muscle from +/*skrr*<sup>m1</sup> neonates (Fig. 4a). The amplitude of the twitches was reduced



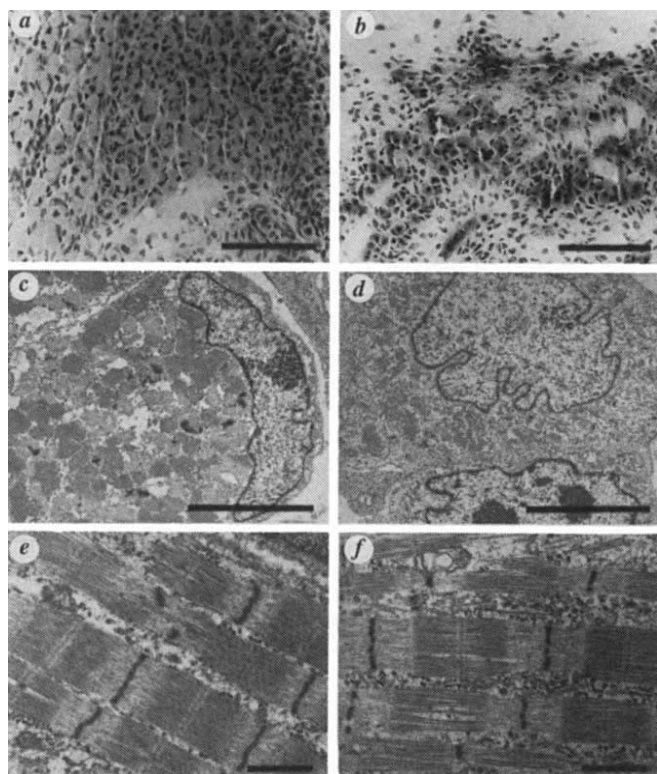
then selected with drug applications (400 µg ml<sup>-1</sup> G418 and 200 nM FIAU). DNA prepared from the resulting ES clones was screened by Southern blot analysis<sup>28</sup> after *Hind*III digestion using the ~0.75 kb *Hind*III-*Bam*HI fragment derived from λMSkRRG6 as a probe. To confirm further the result obtained, other restriction enzymes (*Kpn*I and *Sac*I) and a probe derived from the neomycin-resistance gene were used. ES clones containing the targeted gene were injected into 3.5-day C57B6/J blastocysts<sup>29</sup> and the male chimaeric mice obtained were crossed with female C57B6/J mice to yield mice heterozygous for the *skrr*<sup>m1</sup> mutation. The genotypes of pups obtained by crosses between +/*skrr*<sup>m1</sup> mice were determined by Southern blot and/or PCR analysis<sup>30</sup>. Three lines of *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> mice established from the ES clones 136, 160 and 175 were indistinguishable in perinatal lethality and shared the gross anatomical defects.

**FIG. 2** Analysis of RNA and protein products derived from skeletal muscle ryanodine receptor gene with *skrr*<sup>m1</sup> mutation. **a**, Blot hybridization analysis of total RNA preparations (20 µg each) from hindlimbs of wild-type (+/+), +/*skrr*<sup>m1</sup> (+/-) and *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> (-/-) neonates using a probe for the skeletal muscle ryanodine receptor. The size markers (RNA ladder, BRL) are indicated in kilobases. **b**, RT-PCR analysis of skeletal muscle ryanodine receptor mRNA from wild-type mice (+/+), and from heterozygous (+/-) and homozygous mutant (-/-) hindlimbs. Primers used are indicated in Fig. 1a as FP (forward primer) and RP (reverse primer). The size markers are indicated in basepairs. 221- and 1,413-bp amplified products are expected from the wild-type and mutated transcripts, respectively. **c**, Immunoblotting analysis of microsomal preparations from hindlimbs using polyclonal antibody against the rabbit skeletal muscle ryanodine receptor. The JTC fraction (3 µg protein) from rabbit skeletal muscle (JTC) and microsomal preparations (120 µg protein) from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) neonate hindlimbs were analysed. Size markers (prestained protein standards, BRL) are given in apparent *M<sub>r</sub>*.

**METHODS.** **a**, Total RNA was prepared<sup>31</sup> from skinned lower limbs of neonates and the samples were analysed<sup>32</sup>. The probe used was the ~3.2 kb cDNA insert derived from λMSKRR6. **b**, RT-PCR was done<sup>30</sup> using the total RNA samples and the primers (forward: CCGCCCTCAGGTTCCCGCCAAGCC, reverse: GCGGTTGCCGAACCCCTC-AGCGGCC). An equivalent volume of each reaction mixture was analysed on a 1.8% agarose gel. Partial sequence analysis of the ~1.4 kb amplified cDNA in the lane -/- indicated that the hybridizable RNA species from mutant muscle in northern analysis carries the translation-termination codons and the neomycin-resistance gene on the expected sequence from the targeting construct. The ~1.4 kb band was not



observed in the lane +/-; this may be due to preferential amplification of the smaller fragment, because an RNA species derived from the mutant gene was detected in heterozygous mutant mice by another RT-PCR experiment using the forward primer described above and a reverse primer from the neomycin-resistance gene. **c**, The JTC fraction from rabbit skeletal muscle and microsomal samples from neonate hindlimbs were prepared as previously described<sup>33</sup>. Samples were electrophoresed on an SDS 5% polyacrylamide gel and analysed as described previously<sup>34</sup>, except that rat polyclonal antibody was used after affinity purification and that the rat antibody was detected using peroxidase-conjugated goat antibody to rat IgG (Cappel) and a chemiluminescence reagent (Renaissance, Dupont).



**FIG. 3** Morphological features of skeletal muscle from +/*skrr*<sup>m1</sup> (**a**, **c**, **e**) and *skrr*<sup>m1</sup>/*skrr*<sup>m1</sup> (**b**, **d**, **f**) neonates. Frozen transverse sections (thickness ~10 µm) were stained with haematoxylin and eosin (**a**, **b**). Electron micrographs were obtained from transverse (**c**, **d**) and longitudinal (**e**, **f**) sections. The hindlimb muscles were fixed in 2.5% glutaraldehyde, post-fixed with 2% OsO<sub>4</sub>, enblock stained with saturated uranyl acetate, dehydrated with ethanol, and embedded in Epon. Thinner sections (<50 nm) stained with uranyl and lead were observed and photographed under an electron microscope (JEM-2000EX, JEOL, Japan) at 80 kV. Scale bars, 100 µm (**a**, **b**), 5 µm (**c**, **d**) and 1 µm (**e**, **f**). We did not observe any differences in the morphological features of skeletal muscle between wild-type and +/*skrr*<sup>m1</sup> mice.



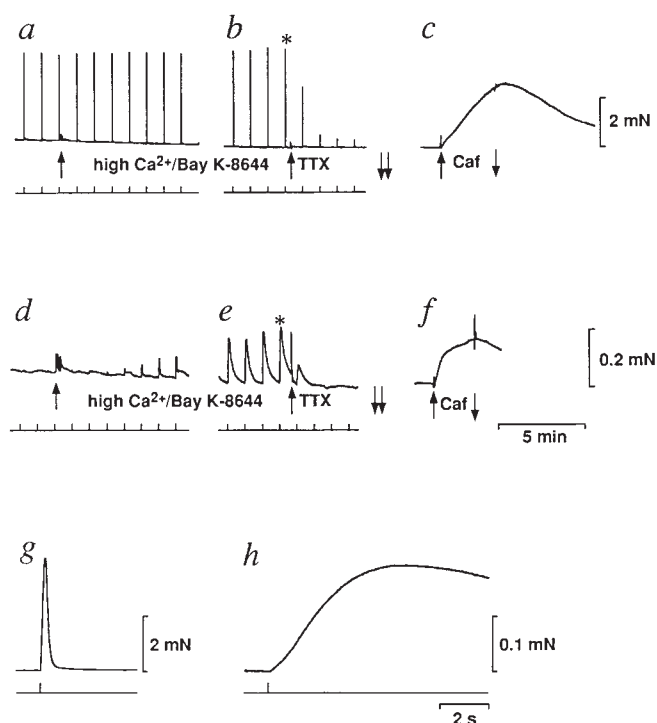


FIG. 4 Comparison of contractile activities in skeletal-muscle preparations from  $+/skrr^{m1}$  (a–c, g) and  $skrr^{m1}/skrr^{m1}$  (d–f, h) neonates. Three parts from a continuous record are shown in each preparation. Isometric tension changes (upper trace) evoked by electrical stimuli (lower trace) in PSS (a, d) or in 12 mM  $Ca^{2+}$  and 10  $\mu$ M Bay K-8644 containing PSS (a, b, d, e). The contractile responses to electrical stimuli were inhibited by 10  $\mu$ M TTX (b, e). Caffeine (25 mM) induced contraction in PSS (c, f). Additions were made at the upward arrows and removals at the downward arrows. The tension responses marked with an asterisk in b and e are shown on a faster time base in g and h, respectively. Note the difference in the tension and time calibrations. The low magnitude of contractions in mutant muscle seems to correlate reasonably well with the deficiency of contractile filaments estimated from the histological observations and RNA blot hybridization analysis. The latter suggested that content of myosin light chain mRNA per limb in mutant mice was roughly an order of magnitude lower than control. The contraction profile of  $+/skrr^{m1}$  muscle was indistinguishable from that in wild-type muscle. METHODS. It was difficult to isolate a muscle intact with its tendons at both ends from  $skrr^{m1}/skrr^{m1}$  mice because of its fragility. We therefore used the entire muscle in the forearm after removal of the skin. The muscle preparations were tied with silk filament at the wrist and the humerus, and were mounted between a pair of stainless-steel rods that were attached to a force transducer (AE801, Akers, Norway) and a micromanipulator (Narishige, Japan). The muscle preparation was immersed in PSS containing (in mM): 150 NaCl, 4 KCl, 2  $CaCl_2$ , 1  $MgCl_2$ , 5 HEPES, 5.6 glucose, pH adjusted to 7.4 with NaOH. Supramaximal field electrical stimulation (20 V, 0.5 ms duration) was delivered by a pair of platinum wires placed on both sides of the preparation. Drug applications were made by replacing the bath solution.

dramatically by the application of tetrodotoxin (TTX), indicating that the twitches were evoked by sodium-channel-dependent action potentials as in normal E-C coupling (Fig. 4b). In contrast, skeletal muscle from  $skrr^{m1}/skrr^{m1}$  mice failed to respond to electrical stimulation in PSS (Fig. 4d). Neither did it show contractile response to depolarization of the sarcolemma by the application of a high- $K^+$  (80 mM) solution (not shown). However, contractions with extremely slow kinetics developed when the same electrical shocks were given in PSS containing 12 mM  $Ca^{2+}$  and 10  $\mu$ M Bay K-8644, an agonist of voltage-gated L-type  $Ca^{2+}$  channels (Fig. 4b versus e; g versus h). These slow contractions were abolished by the application of TTX (Fig. 4e),

indicating that action potentials are generated in the mutant muscle but that  $Ca^{2+}$  release after depolarization of sarcolemma is impaired in the mutant muscle. Thus, our results indicate strongly that the ryanodine receptor functions as the  $Ca^{2+}$  release channel in the skeletal-muscle E-C coupling as proposed previously<sup>3–6</sup>.

The slow contraction after the electrical stimulation in mutant muscle might be due to slow influx of  $Ca^{2+}$  through L-type  $Ca^{2+}$  channels, which directly activated the contractile proteins. However, this seems rather unlikely because high  $Ca^{2+}$  concentration and Bay K-8644 had no effect on the twitch contractions in muscle from  $+/skrr^{m1}$  mice (Fig. 4a, b). Alternatively, the  $Ca^{2+}$ -induced  $Ca^{2+}$  release from the SR after enhanced  $Ca^{2+}$  influx might contribute to the contraction. Indeed, skeletal muscle from the mutant mice seems to retain the ability to release  $Ca^{2+}$  in response to caffeine (Fig. 4f). Thus, ryanodine receptors other than the skeletal muscle type seem to contribute to  $Ca^{2+}$  release from the sarcoplasmic reticulum in the mutant muscle. Assuming that either the cardiac or the brain ryanodine receptor was expressed in the mutant muscle, our results suggest that the expressed receptor type cannot substitute for the skeletal muscle ryanodine receptor in the physiological E-C coupling of skeletal muscle. By contrast, the skeletal muscle DHP receptor, which functions as the voltage sensor during E-C coupling<sup>22,23</sup>, is expressed in the mutant muscle as suggested by northern analysis (data not shown). Thus, these observations imply that not only the DHP receptor<sup>24</sup>, but also the type of ryanodine receptor, determines whether E-C coupling is skeletal muscle or cardiac type. The function of the ryanodine receptor at the molecular level remains to be further investigated. The  $skrr^{m1}/skrr^{m1}$  mouse may provide an important system for the investigation of the role of the ryanodine receptor molecule during E-C coupling. □

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# Transport of cytoplasmic particles catalysed by an unconventional myosin in living *Drosophila* embryos

Valerie Mermall\*, James G. McNally\*† & Kathryn G. Miller\*

\*Department of Biology and †Institute for Biomedical Computing, Washington University, St Louis, Missouri 63130, USA

MYOSINS are actin-activated ATPases that are able to translocate along actin filaments using energy derived from ATP hydrolysis. Non-muscle cells contain conventional myosins, which are similar in sequence and structure to muscle myosin, and a number of unconventional myosins whose head sequences are similar but tail sequences are unrelated to conventional myosins<sup>1</sup>. The myosin superfamily currently consists of nine classes<sup>2</sup>; *Drosophila* 95F is an unconventional myosin<sup>3</sup> and the original member of class VI, which includes a homologue found in pig kidney<sup>2</sup>. Some unconventional myosins have been suggested as mediators of some types of intracellular transport<sup>4,5</sup>, but there is little direct evidence for this function (but see ref. 6). We have observed transport of cytoplasmic particles in live *Drosophila* embryos in three dimensions using computational optical sectioning microscopy. We present here evidence that this transport is actin-based, ATP-dependent and catalysed by one such unconventional myosin, the 95F myosin. This is, to our knowledge, the first direct observation of transport catalysed by an unconventional myosin in living cells.

We observed the motion of 95F myosin-containing particles in three dimensions in living cells by injecting rhodamine-labelled monoclonal antibody 3C7 (Rd-mAb 3C7) into syncytial blastoderm *Drosophila* embryos. A region of cytoplasm immediately under the plasma membrane was imaged using fluorescence time-lapse computational optical-sectioning microscopy<sup>7</sup>. Antibody 3C7 is specific for the 95F myosin; when injected into live embryos, it incorporates into the same particulate structures (Fig. 1) as seen in fixed preparations<sup>3</sup> but, at the concentrations used in this study, it does not cause developmental defects (K.G.M., unpublished observations). The antibody determinant resides in the tail of the molecule.

Observation of both fixed specimens and time-lapse recordings demonstrates that the particles undergo cell cycle-coordinated rearrangements. During interphase the particles are distributed throughout the cytoplasm (Fig. 1a); at metaphase they move into transient invaginations of the plasma membrane called pseudocleavage furrows (Fig. 1b). To determine if these movements are the result of an active process, *X*, *Y* and *Z* coordinates were measured for selected particles in a sequence of time points. Three-dimensional representations of the particle paths are displayed in Fig. 2a. Of the particles (Table I), 29% move in a linear fashion (at least 5 sequential steps in one direction; Fig. 2a), generally following relatively straight paths for more than 75 s. Other particles move along random trajectories (Fig. 2a). Particles typically move into and out of the small volume imaged during the recording, generally remaining visible for 1–3 min (total of 5 to 20 time points). Particles sometimes move, stop, then move again during a time-lapse sequence and move with a variable velocity. The average speed of particle movement is  $0.03 \mu\text{m s}^{-1}$  (data from 14 particles, 123 steps, s.d. =  $0.02 \mu\text{m s}^{-1}$ ). This *in vivo* velocity is within the range of previously measured *in vitro* velocities ( $0.01 \mu\text{m s}^{-1}$  for *Acanthamoeba* myosin IA<sup>8</sup> to  $0.2 \mu\text{m s}^{-1}$  for *Acanthamoeba* organelles<sup>4</sup>) for unconventional myosin-mediated motility in the *Nitella*<sup>9</sup> assay. The linear movement of particles is very different from the movement of freely diffusing latex microspheres injected into the cortex of *Drosophila* embryos (Fig. 2a). The microspheres move

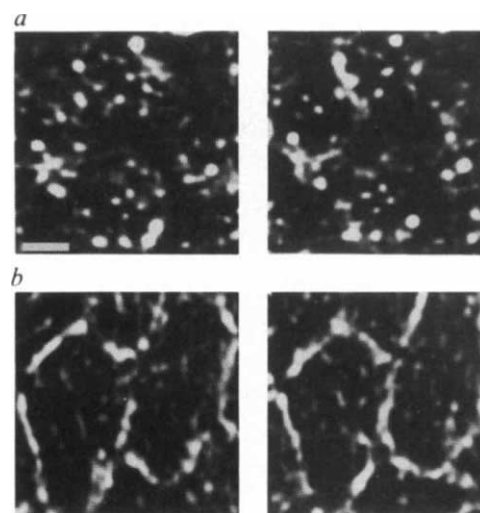


FIG. 1 Stereo-pair fluorescence micrographs of living *Drosophila* embryos microinjected with rhodamine-labelled monoclonal antibody against 95F myosin (Rd-mAb 3C7). a, Distribution of 95F myosin-containing particles during interphase, showing that they are distributed in domains within the cortical cytoplasm. b, Distribution of 95F myosin-containing particles during mitosis, showing concentration in the pseudocleavage furrows.

METHODS. *Drosophila* Oregon R syncytial blastoderm embryos were microinjected by standard techniques<sup>19</sup> with tracer amounts of Rd-mAb 3C7. Embryos were incubated for 20 min before optical sectioning microscopy. Thirty sections,  $0.5 \mu\text{m}$  apart were collected from the cortical region using an Olympus IM2 inverted fluorescence microscope equipped with a CCD camera. Focal plane position and image acquisition were controlled by a computer. The fluorescence intensity of each section was scaled to correct for lamp flicker, background fluorescence was subtracted, and two-dimensional gaussian filtering (D. A. Agard and J. W. Sedat, personal communication) was done to remove noise and enhance contrast. Stereo-pair projections were calculated at  $7.5^\circ$ . These images show a  $35 \times 35 \times 15 \mu\text{m}$  region of cytoplasm from syncytial blastoderm embryos that measure  $\sim 500 \mu\text{m}$  in length and  $100 \mu\text{m}$  in diameter. Scale bar,  $3 \mu\text{m}$ .

TABLE 1 Numbers of particles undergoing linear motion

| Treatment      | Number of particles<br>Linear motion | Total | %<br>Linear* | Min of observa-<br>tion† | Persist-<br>ence‡ |
|----------------|--------------------------------------|-------|--------------|--------------------------|-------------------|
| Untreated      |                                      |       |              |                          |                   |
| (2)            | 95                                   | 232   | 29           | 56                       | 0.63              |
| DNP (4)        | 4                                    | 161   | 3            | 42                       | 0.27              |
| CytoD (2)      | 4                                    | 100   | 4            | 42                       | 0.31              |
| pAb (4)        | 7                                    | 198   | 4            | 110                      | 0.30              |
| Colchicine (4) | 53                                   | 277   | 19           | 48                       | ND                |

Stereo-pair movies were examined to determine the total number of particles and the number of particles moving along linear paths. A particle was scored as undergoing linear motion if it moved along a linear path for at least 5 steps. Movies were prepared and inhibitor treatments were as in Fig. 2. The number of independent recordings scored for linear motion is shown in parentheses.

\* In the case of embryos injected with DNP, cytochalasin D (cytoD), and polyclonal antibody (pAb), individual particles remain visible for much longer periods of time than in labelled embryos without inhibitors. Thus, each particle counted in inhibitor-treated embryos is observed for much longer periods of time than transported particles. These numbers are not normalized for observation time.

† This is the total length of developmental time recorded in the movies over which these counts were made.

‡ Persistence is the ratio of displacement to total path length. The persistence values for particles from DNP-, cytoD-, and pAb-injected embryos are consistent with random motion as they are similar to that obtained in a random walk (0.36), and are distinct from the persistence of particles undergoing linear motion.

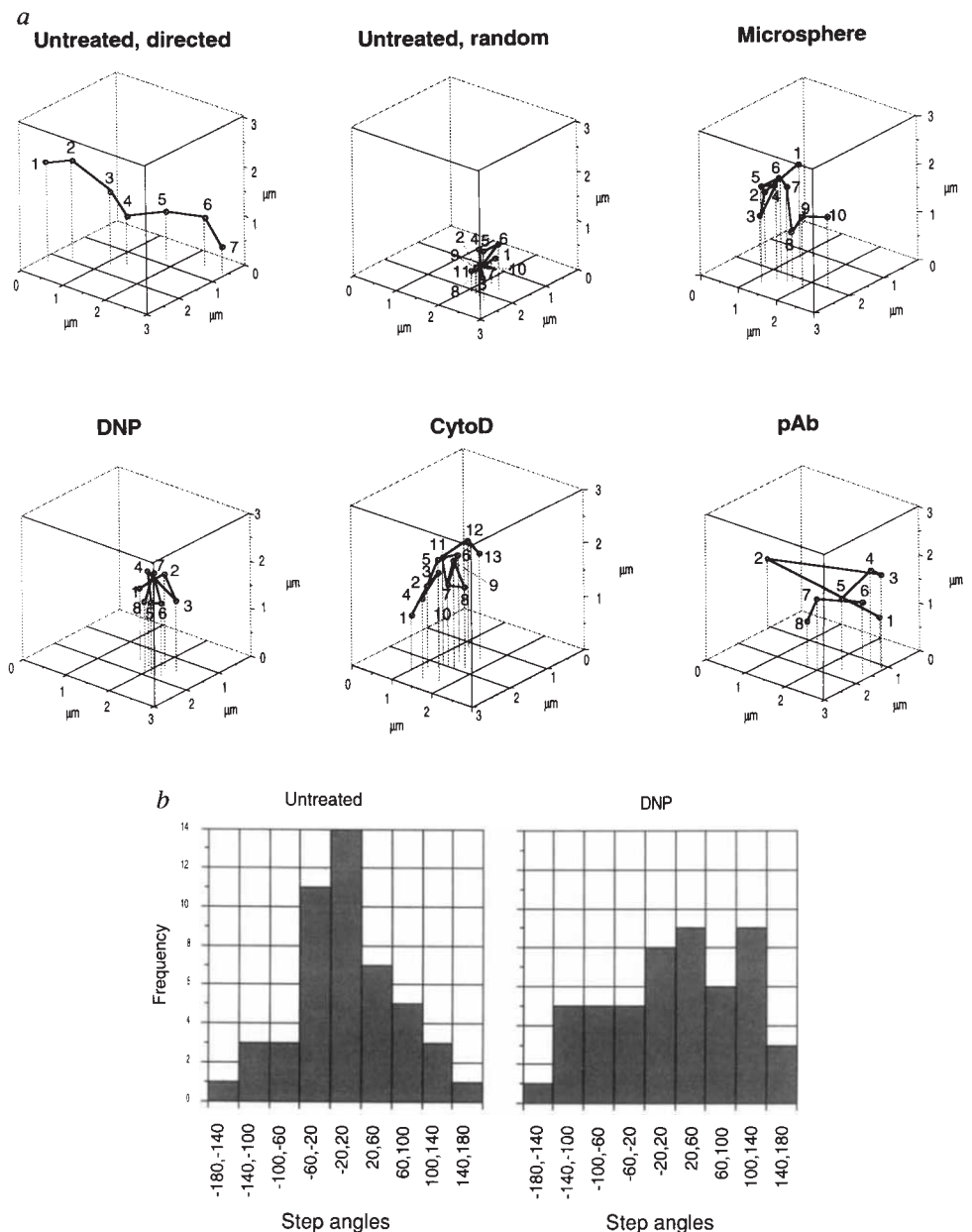


FIG. 2 a, Three-dimensional plots of particle coordinates from a series of time-lapse images. The plots in the top row represent two different behaviours of cytoplasmic particles in labelled embryos without inhibitors, and the behaviour of  $0.3\text{ }\mu\text{m}$  latex spheres. In the second row, behaviour of particles in inhibitor-injected embryos is represented. The motion of particles in cytochalasin D- and pAb-treated embryos is similar to the free diffusion of the microspheres; in contrast, randomly moving particles in untreated and DNP-injected embryos are similar to each other, but distinct from the movement of microspheres. b, Analysis of the angles between successive steps for particles from untreated and DNP-treated embryos. Particles moving randomly are expected to have a random distribution of step angles, whereas particles undergoing linear motion should display a bias in step angles<sup>11</sup>. This 2D analysis includes only those paths with no Z component, avoiding complications from the different spatial resolutions between XY ( $0.137\text{ }\mu\text{m}$ ) and Z data ( $0.5\text{ }\mu\text{m}$ ). We used the Z statistic<sup>20</sup> to assess the uniformity of step angle distribution. This analysis confirms uniformity in all treatments which disrupt linear motion ( $Z=0.10$  for pAb, 1.2 for cytoD, and 2.0 for DNP-treated embryos, where  $Z<2.9$  is uniform at the 5% significance level), and allows us to reject the hypothesis of uniformity in the case of linear motion ( $Z=10.5$ , where  $Z<4.6$  is uniform at the 1% significance level). An analysis of 3D step angles<sup>21</sup>, which accounts for the bias in spatial resolution, supports the 2D results.

**METHODS.** Embryos were injected with Rd-mAb 3C7 and time-lapse optical sectioning microscopy was done by collecting one 3D image (a  $17.5\times 17.5\times 5\text{ }\mu\text{m}$  volume), consisting of 10 sections,  $0.5\text{ }\mu\text{m}$  apart, every 15 s. The images were processed as in Fig. 1 and stereo-pair movies were examined to assess particle movement. The 3D coordinates of the particles were determined by locating a particle within the optical sections of a given time point and determining its centroid position (K. Doolittle, personal communication). Fluorescent red  $0.3\text{ }\mu\text{m}$  carboxylate latex microspheres (Molecular Probes) were blocked with 0.1% BSA in PBS, before dilution with PBS and injection. Fifty labelled embryos without inhibitors were examined. In inhibitor experiments, embryos were injected with DNP ( $n=8$ ;  $200\text{ }\mu\text{g ml}^{-1}$ ,  $\sim 21\text{ }\mu\text{M}$  final concentration), cytochalasin D ( $n=8$ ;  $200\text{ }\mu\text{g ml}^{-1}$ ,  $\sim 7.9\text{ }\mu\text{M}$  final concentration), anti-95F myosin polyclonal antibody ( $n=17$ ;  $300\text{ }\mu\text{g ml}^{-1}$ ,  $\sim 38\text{ nM}$  final concentration), anti-glutathione-S-transferase polyclonal control antibody ( $n=4$ ;  $400\text{ }\mu\text{g ml}^{-1}$ , final concentration  $\sim 50\text{ nM}$ ) or colchicine ( $n=7$ ;  $2\text{ mg ml}^{-1}$ ,  $\sim 100\text{ }\mu\text{M}$

faster and more erratically than particles undergoing transport. These  $0.3\text{ }\mu\text{m}$  spheres are roughly the same size as the fluorescence image of the cytoplasmic particles and represent an upper size limit for the particles.

If the linear motion of cytoplasmic particles is due to active transport mediated by a myosin, it should require ATP and actin



final concentration; data not shown) 20 min. after labelling. Time-lapse optical sectioning was done 5–80 min after the inhibitor injection. In the case of DNP-, cytoD- and colchicine-injected embryos, embryonic development was arrested at these concentrations of inhibitors. In DNP-injected embryos, particle transport ceases within 5 min whereas the distribution of actin filaments, as visualized by phalloidin staining of embryos fixed after DNP injection, remains substantially undisturbed for at least 1 h (data not shown). In cytoD-injected embryos that were fixed and stained with phalloidin 20 min after drug injection, normal actin structures were disrupted and only actin aggregates and occasional short actin filaments were visible. In colchicine-treated embryos fixed and stained with anti-tubulin antibody 20 min after injection, no microtubules were visible.

filaments. We injected dinitrophenol (DNP), an inhibitor of ATP production in *Drosophila*<sup>10</sup>, into labelled embryos. Particles in DNP-injected embryos move randomly and stay in the field of view longer than particles in untreated embryos; 96% of the particles undergo random movement, whereas a maximum of 4% of observed particles undergo movement consistent with

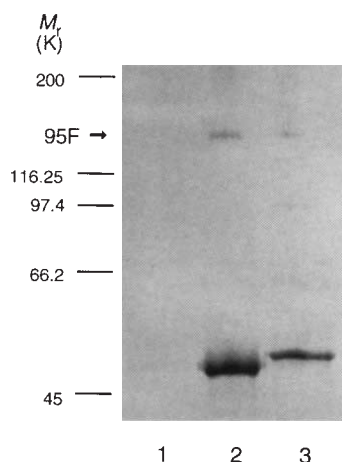


FIG. 3 Specificity of the polyclonal and monoclonal antibodies as assessed by immunoprecipitation. A Coomassie blue-stained SDS-polyacrylamide gel of proteins immunoprecipitated by monoclonal antibody 3C7 and the polyclonal antiserum against 95F myosin is shown. Lane 1, no antibody control; lane 2, immunoprecipitation with affinity-purified polyclonal antibody; lane 3, immunoprecipitation with mAb 3C7.  $M_r$  and the position of 95F myosin are indicated on the left. The 95F myosin is visible in immunoprecipitates with either the polyclonal and monoclonal antibody. The monoclonal antibody immunoprecipitates one additional minor protein at 170K which is not present when the polyclonal antibody is used. The heavily stained low  $M_r$  bands are antibody heavy chains. METHODS. Monoclonal antibody 3C7 culture supernatant, affinity-purified polyclonal anti-95F myosin antibody or PBS was incubated overnight at 4 °C with 25  $\mu$ l protein A agarose beads (BioRad). After washing with IP buffer (20 mM HEPES, 150 mM NaCl, 250 mM sucrose, pH 7.5) the beads were incubated overnight at 4 °C with clarified extract prepared from 1 g of 0–3h *Drosophila* embryos in 5 ml of IP buffer, 1 mM PMSF. The beads were washed with IP buffer, 350 mM KCl and analysed by SDS-PAGE. For actin-binding experiments, F-actin (final concentration 9  $\mu$ M) was added to 95F myosin immobilized on protein A beads by mAb 3C7, with or without excess (1.8  $\mu$ M) polyclonal antibody, incubated on ice for 30 min and spun through a 30%, 60% sucrose step gradient in IP buffer. Proteins on the beads were fractionated by SDS-PAGE and stained with Coomassie blue. The relative amounts of actin and 95F myosin which pelleted with the beads were determined by scanning gels with a Hewlett-Packard ScanJet Plus scanner and quantified with NIH Image software.

transport (Fig. 2a, Table 1). Particles in DNP-treated embryos and particles moving randomly in untreated embryos move through less volume than 0.3  $\mu$ m microspheres injected into the cortex of embryos (Fig. 2a). This suggests that these particles are attached to a larger, more slowly diffusing structure, such as actin filaments. When actin is depolymerized in labelled embryos by treatment with cytochalasin D, only 3% of the total particles move along linear paths (Fig. 2a, Table 1). Treatment of embryos with colchicine does not stop the linear motion (Table 1), despite the depolymerization of microtubules in these embryos (data not shown).

To determine if 95F myosin is the motor responsible for linear particle motility we used anti-95F myosin polyclonal antibody. The specificity of this antibody was demonstrated by immunoblotting<sup>3</sup> and immunoprecipitation experiments (Fig. 3, and V. Lantz, unpublished observations). This polyclonal antibody inhibits the *in vitro* actin-binding activity of 95F myosin. In four experiments, the antibody inhibited between 50–82% of actin-binding activity (67% average, s.d. = 16). When labelled embryos are injected with polyclonal antibody, only 3% of the total particles undergo linear movement (Fig. 2a, Table 1). The injected polyclonal antibody (~0.04  $\mu$ M, final) does not saturate

the available 95F myosin (~0.5  $\mu$ M), and thus produces a concentration gradient. We observe a disruption of linear motion near the injection site; normal particle motility is observed further away. Because we are using less than saturating amounts of this specific polyclonal antibody in our injection experiments, it is unlikely that it is affecting a protein other than 95F myosin. The motility of particles in anti-95F myosin antibody and cytochalasin D-injected embryos closely resembles the free diffusion of the injected microspheres, suggesting that 95F myosin-associated particles are undergoing free diffusion in polyclonal antibody-injected embryos, consistent with the antibody inhibition of actin-binding. Injections of a control polyclonal antibody against glutathione-S-transferase had no effect on transport (data not shown).

To assess quantitatively the linear nature of particle trajectories, we measured the angles between adjacent steps along trajectories (step angles) and determined the persistence of linear motion. If the particles are moving randomly, the step angles should be uniformly distributed<sup>11</sup>. For particles undergoing linear motion, the distribution of step angles is non-uniform; step angle distribution is not significantly different from random in all other cases (Fig. 2b). Persistence, the ratio of displacement over total path length, is 1 for a particle moving in a perfect line, and approaches 0 for a wandering particle<sup>12</sup>. Paths of linearly moving particles from untreated embryos have high persistence values, unlike those of particles undergoing other types of motion (Table 1).

Our results demonstrate that the linear motion of 95F myosin-containing particles requires ATP and occurs on actin filaments, consistent with myosin-based transport; and suggests the 95F unconventional myosin as the motor. Unconventional myosins have previously been suggested as motors for the transport of cytoplasmic components such as vesicles from localization studies in a variety of cell types<sup>13–15</sup>, cell fractionation data<sup>4,16,17</sup>, and the phenotypes of cells with mutations in myosin genes<sup>5,18</sup>. In addition, myosin-mediated transport of vesicles has been demonstrated *in vitro*<sup>4</sup>. Our observations demonstrate that the 95F unconventional myosin is a motor that transports cytoplasmic particles along actin filaments *in vivo*. This transport results in the redistribution of the particles in a cell-cycle dependent manner. □

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# Cloned $\text{Ca}^{2+}$ -dependent $\text{K}^+$ channel modulated by a functionally associated protein kinase

Manuel Esguerra\*, Jing Wang\*,  
Christine D. Foster\*†, John P. Adelman‡,  
R. Alan North†† & Irwin B. Levitan\*§

\* Department of Biochemistry and Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02254, USA

‡ Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201, USA

† Present addresses: Department of Physiology, Glaxo Inc., 5 Moore Drive, Research Triangle Park, NC 27709, USA (C.D.F.); Glaxo Institute for Molecular Biology, 1228 Plan-les-Ouates, Geneva, Switzerland (R.A.N.)

§ To whom correspondence should be addressed

**CALCIUM-DEPENDENT potassium ( $\text{K}_{\text{Ca}}$ ) channels carry ionic currents that regulate important cellular functions<sup>1-3</sup>. Like some other ion channels<sup>4-10</sup>,  $\text{K}_{\text{Ca}}$  channels are modulated by protein phosphorylation<sup>2,11,15-21</sup>. The recent cloning of complementary DNAs encoding *Slo*  $\text{K}_{\text{Ca}}$  channels<sup>12-14</sup> has enabled  $\text{K}_{\text{Ca}}$  channel modulation to be investigated. We report here that protein phosphorylation modulates the activity of *Drosophila Slo*  $\text{K}_{\text{Ca}}$  channels expressed in *Xenopus* oocytes. Application of ATP- $\gamma\text{S}$  to detached membrane patches increases *Slo* channel activity by shifting channel voltage sensitivity. This modulation is blocked by a specific inhibitor of cyclic AMP-dependent protein kinase (PKA). Mutation of a single serine residue in the channel protein also blocks**

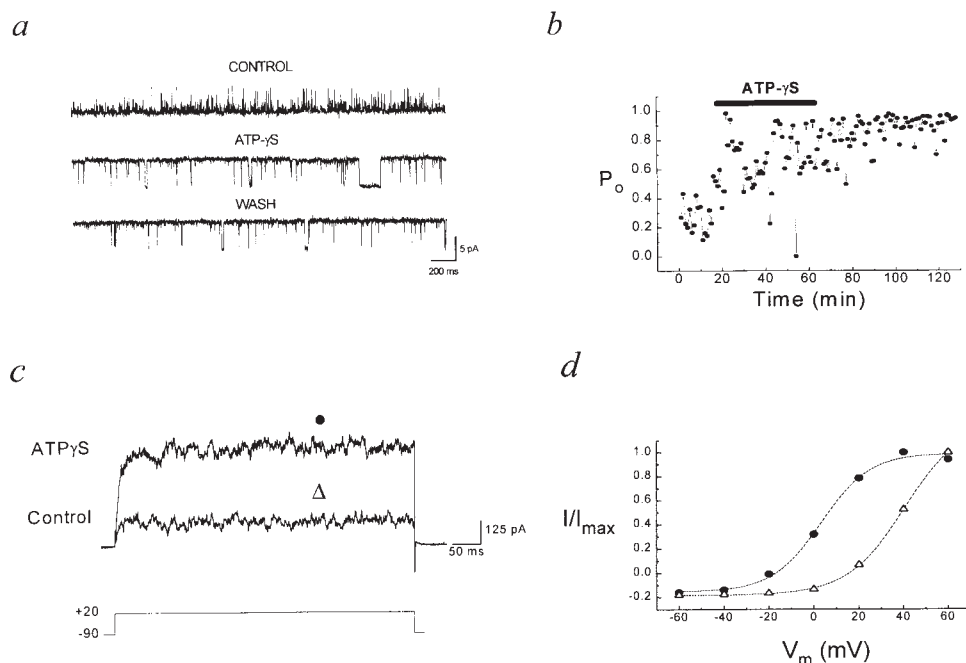
modulation by ATP- $\gamma\text{S}$ , demonstrating that phosphorylation of the *Slo* channel protein itself modulates channel activity. The results also indicate that  $\text{K}_{\text{Ca}}$  channels in oocyte membrane patches can be modulated by an endogenous PKA-like protein kinase which remains functionally associated with the channels in the detached patch.

To investigate whether phosphorylation by exogenous PKA can modulate the activity of *Drosophila Slo* channels, we examined the effects of this kinase on the channel's steady-state behaviour. Application of the catalytic subunit of PKA in the presence of ATP upregulates channel open probability without affecting single-channel conductance ( $n=4/5$  patches; data not shown).

Previous experiments have shown that at least one type of reconstituted  $\text{K}_{\text{Ca}}$  channel from rat brain can also be modulated by ATP or ATP- $\gamma\text{S}$  alone in the absence of exogenous protein kinase, indicative of phosphorylation by a protein kinase activity that remains closely associated with reconstituted channels in artificial lipid bilayers<sup>19</sup>. We therefore examined the effects of ATP and ATP- $\gamma\text{S}$  on *Slo* channels in detached patches from oocytes. Our initial experiments showed that ATP alone both increases and decreases *Slo* channel activity. Application of phosphatase inhibitors suggested that the decrease in activity is a complex phenomenon involving a phosphoprotein phosphatase activity that comes away with the detached patch (see also ref. 22). To eliminate this complication, we used ATP- $\gamma\text{S}$  in the following experiments, because thiophosphorylated residues in proteins are poor substrates for phosphatases<sup>23</sup>. We found that addition of ATP- $\gamma\text{S}$  alone always increased the activity of *Slo* channels expressed in oocytes ( $n=22/23$ ; Fig. 1a, b). This effect persists after washout of ATP- $\gamma\text{S}$ , which is consistent with covalent modification of a protein or proteins in the patch, rather than with direct binding effects of ATP- $\gamma\text{S}$  (Fig. 1a, b). In support of this, application of adenylylimidodiphosphate (AMP-PNP), a non-hydrolysable analogue of ATP, does not modulate

FIG. 1 Modulation of *Slo* by ATP- $\gamma\text{S}$ . a, Record of  $\text{K}_{\text{Ca}}$  channel activity, in the absence and presence of 200  $\mu\text{M}$  ATP- $\gamma\text{S}$ , in an inside-out oocyte patch excised 32 h after injection of *Slo* cRNA. Free calcium ( $[\text{Ca}^{2+}]_{\text{in}}$ ) 200  $\mu\text{M}$ , patch potential +20 mV. b, Open probability ( $P_o$ ) of the channel in a increases within 5 min after addition of ATP- $\gamma\text{S}$ , with no change in single-channel conductance. The effect does not reverse for up to 1 h after washout of ATP- $\gamma\text{S}$ . c, Macroscopic currents recorded from many *Slo* channels in an inside-out patch 8 days after RNA injection, in the absence and presence of 200  $\mu\text{M}$  ATP- $\gamma\text{S}$ . Currents were evoked by voltage steps from -90 mV to pulse potentials between -60 and +75 mV in the presence of 200  $\mu\text{M}$   $[\text{Ca}^{2+}]_{\text{in}}$ . Representative curves at a pulse potential of +20 mV are shown. Symbols indicate time points used to construct normalized current-voltage curves (d). The curve is shifted to the left by ATP- $\gamma\text{S}$ , indicating that the channel's voltage sensitivity has increased.

**METHODS.** *Xenopus* oocytes were injected with mRNA transcribed *in vitro* from cDNA coding for *Slo* variant A1C2E1G3I0 (ref. 13). Single-channel and macroscopic currents were recorded using standard methods from inside-out patches excised 18 h to 14 d after RNA injection. Total and free  $\text{Ca}^{2+}$  concentrations were calculated using the Chelator program<sup>28</sup> and verified by Fura fluorescence.  $\text{Cl}^-$  was excluded from bath solutions to prevent contamination by endogenous  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. The pipette (external) solution contained (mM):



$\text{K}^+$  gluconate, 2.5;  $\text{Na}^+$  gluconate, 115;  $\text{Ca}^{2+}$  gluconate 1.8; HEPES 10, pH 7.2. Intracellular (bath) solution contained (mM):  $\text{K}^+$  gluconate 116;  $\text{Mg}^{2+}$  gluconate 2.0; EGTA 0.1; HEPES 10, pH 7.2. Channel open probabilities were determined by integrating the area under an all-points amplitude histogram. *Drosophila Slo* channels resembled other maxi- $\text{K}_{\text{Ca}}$  channels<sup>29</sup> in their large single-channel conductance (123 pS) and dependence on internal  $\text{Ca}^{2+}$  and transmembrane voltage.

FIG. 2 A specific PKA inhibitor blocks ATP- $\gamma$ S modulation of *Slo* channels. *a*, In the presence of inhibitory peptide PKI<sub>6-22</sub> (50–100 nM; Gibco) applied to the internal face of the patch, ATP- $\gamma$ S applied for up to 20 min has no effect on *Slo* channel activity. Application of PKI<sub>6-22</sub> alone does not affect channel kinetics or conductance. *b*, Plot of channel open probability as a function of time for two different channels (open and filled circles) with different basal open probabilities, showing that there is no effect of ATP- $\gamma$ S in the presence of PKI<sub>6-22</sub>. Filled circles are from the channel shown in *a*.

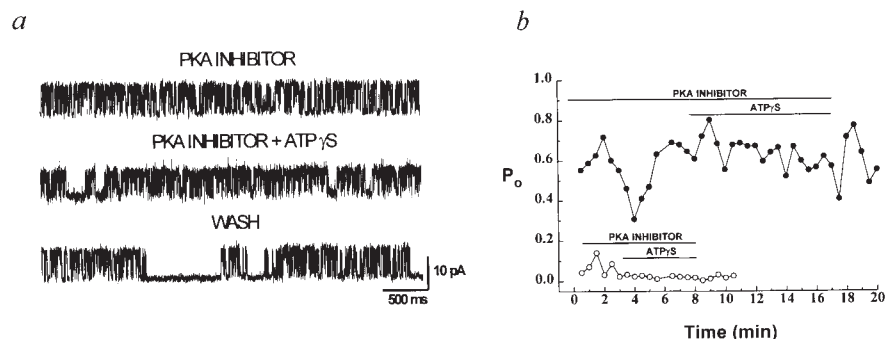
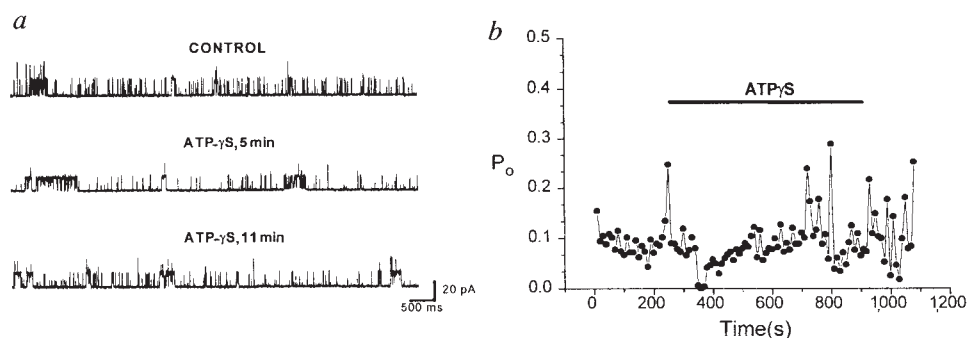


FIG. 3 S942A *Slo* mutant is not modulated by ATP- $\gamma$ S. *a*, Recording from a patch containing several *Slo* channels in which serine 942 was mutated to alanine (S942A). ATP- $\gamma$ S does not alter the activity of such mutant channels. *b*, Plot of channel open probability as a function of time, showing that there is no significant effect of ATP- $\gamma$ S on S942A *Slo* channels. The average open probability of these channels was  $26 \pm 24\%$  lower (mean  $\pm$  s.e.) after ATP- $\gamma$ S treatment; for wild-type channels (Fig. 1) the open probability was  $237 \pm 23\%$  higher after addition of ATP- $\gamma$ S.



METHODS. Serine 942 was mutated to an alanine residue using the polymerase chain reaction (PCR). Oligonucleotide primers coding for the serine-to-alanine mutation and for appropriate restriction sites were used in the PCR to amplify a 330-base-pair fragment containing the putative protein kinase A consensus sequence around S942. The

channel activity ( $n = 2$ ). In some experiments, channel expression was sufficiently high for us to measure macroscopic  $K_{Ca}$  currents in the detached patch (Fig. 1c); ATP- $\gamma$ S markedly increases the current evoked by depolarizing voltage steps in these patches (Fig. 1c), and this effect is due to a leftward shift in the channel voltage sensitivity (Fig. 1d).

The results with ATP analogues suggested a role for protein phosphorylation in the upregulation of *Slo* by ATP- $\gamma$ S. To test this, we used PKI<sub>6-22</sub>, a specific peptide inhibitor of PKA<sup>24</sup>. In the presence of this inhibitor, ATP- $\gamma$ S does not modulate *Slo* channel activity ( $n = 8/8$ ; Fig. 2a, b), implying that modulation in these detached patches depends on protein phosphorylation by a constitutively active, patch-bound, PKA-like protein kinase.

Modulation of *Slo* by such a PKA-like kinase suggests that the channel protein might be phosphorylated on serine or threonine residues. Alternatively, the kinase might phosphorylate some other protein(s) in the patch, which in turn modulate(s) *Slo* open probability. To distinguish between these possibilities, we examined whether particular amino-acid residues in the *Slo* channel protein itself are targets for modification by cAMP-dependent protein kinase. We constructed cDNA coding for a mutant *Slo* in which the serine residue at position 942, which lies within the only consensus sequence for PKA phosphorylation<sup>25</sup> in the channel protein, is replaced by alanine (S942A). A fusion protein containing a fragment of the channel protein (amino acid residues 821–993), including S942, is readily phosphorylated by PKA *in vitro*, whereas the corresponding fragment containing the S942A mutation is not phosphorylated (data not shown), strongly suggesting that S942 is an effective substrate for PKA phosphorylation. Accordingly, we injected oocytes with cRNA-encoding *Slo* channels containing the S942A

amplified mutant fragment was ligated into a modified pGEM vector containing the *Slo* cDNA, replacing the corresponding wild-type fragment. The S942A mutation was confirmed by DNA sequencing. cRNA was transcribed from this construct *in vitro* and injected into *Xenopus* oocytes as described.

substitution. Mutant channels are strongly expressing and show unaltered kinetics or single-channel conductance, indicating that the substitution does not induce significant changes in basal channel activity. However, the mutant channel is not subject to modulation. Application of ATP- $\gamma$ S, either alone or in the presence of PKA catalytic subunit, does not modulate the activity of S942A *Slo* channels ( $n = 5/5$ ; Fig. 3a, b). These results are consistent with the hypothesis that modulation of wild-type channel activity by ATP- $\gamma$ S is due to direct thiophosphorylation of S942 in the channel protein, catalysed by a PKA-like protein kinase that remains functionally associated with the channel in the detached membrane patch.

We demonstrate here that phosphorylation of a specific serine residue in the channel protein can modulate the activity of a cloned  $K_{Ca}$  channel. This result adds to the emerging story<sup>11</sup> of ion-channel modulation by direct phosphorylation of specific amino-acid residues in a variety of different ion channels. Of particular interest is the finding that the modulation described here is due to an endogenous protein kinase activity. Exogenous kinase is not necessary to produce phosphorylation, a result similar to that with rat brain  $K_{Ca}$  channels reconstituted into planar lipid bilayers<sup>19</sup> (but see also ref. 26). The present finding takes on added significance because the channels are expressed in a heterologous system. Remarkably, it appears that an endogenous protein kinase in the host cell not only associates closely with a heterologously expressed ion-channel protein, but retains its ability to modulate channel function in a detached membrane patch. Such submembrane complexing of ion-channel proteins with modulatory enzymes like kinases and phosphatases may be a common means by which cells achieve highly localized regulation of ion-channel function by otherwise ubiquitous biochemical processes<sup>19,22,27</sup>. □



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## Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production *in vivo*

Si Lok\*, Kenneth Kaushansky†, Richard D. Holly\*, Joseph L. Kuijper\*, Catherine E. Lofton-Day\*, Pieter J. Oort\*, Francis J. Grant\*, Mark D. Heipel\*, Steve K. Burkhead\*, Janet M. Kramer\*, L. Anne Bell\*, Cindy A. Sprecher\*, Hal Blumberg\*, Rebecca Johnson\*, Donna Prunkard\*, Andrew F. T. Ching\*, Shannon L. Mathewes\*, Mason C. Bailey\*, John W. Forstrom\*, Michele M. Buddle\*, Sherri G. Osborn\*, Simon J. Evans\*, Paul O. Sheppard\*, Scott R. Presnell\*, Patrick J. O'Hara\*, Fredrick S. Hagen\*, Gerald J. Roth† & Donald C. Foster†\*

\* ZymoGenetics Corporation, 4225 Roosevelt Way NE, Seattle, Washington 98105, USA

† Division of Hematology, University of Washington School of Medicine, Washington 98195, USA

THE major regulator of circulating platelet levels is believed to be a cytokine termed thrombopoietin<sup>1,2</sup>. It is thought to be a lineage-specific cytokine affecting the proliferation and maturation of committed cells resulting in the production of megakaryocytes and platelets. Despite considerable efforts by a number of laboratories, the unequivocal identification of thrombopoietin has proven elusive. Here we report the functional cloning of a murine complementary DNA encoding a ligand for the receptor encoded by the *c-mpl* proto-oncogene (*c-Mpl*)<sup>3–5</sup>. The encoded polypeptide has a predicted molecular mass of 35,000 (*M*, 35K). The protein has a novel two-domain structure with an amino-terminal domain homologous with erythropoietin and a carboxy-terminal domain rich in serine, threonine and proline residues and containing seven potential *N*-linked glycosylation sites. Intraperitoneal injections of mice with recombinant protein increase circulating platelet levels by greater than fourfold after 7 days. These results along with those presented in the accompanying report strongly suggest that the ligand for *c-Mpl* is thrombopoietin.

The *c-mpl* proto-oncogene<sup>3–5</sup> is a newly described member of the haematopoietin receptor superfamily and its function has been implicated in megakaryocytopoiesis<sup>6,7</sup>. To aid the cloning

of a ligand for *c-Mpl*, we constructed a *c-Mpl* ligand-dependent cell line, BaF3/MPLR1.1, for use in a cell proliferation assay for the screening of an expression cDNA library. A mutant cell line, 24-11, which produced *c-Mpl* ligand, was isolated by treating BaF3/MPLR1.1 cells with 2-ethylmethanesulphonate (EMS)<sup>8</sup> and selecting for clones exhibiting exogenous factor-independent autocrine growth. Two essentially identical full-length cDNA clones were isolated from screening ~600,000 recombinants from a cDNA expression library constructed from messenger RNA from 24-11 cells. One cDNA, pZGmpl-1081 was studied in detail.

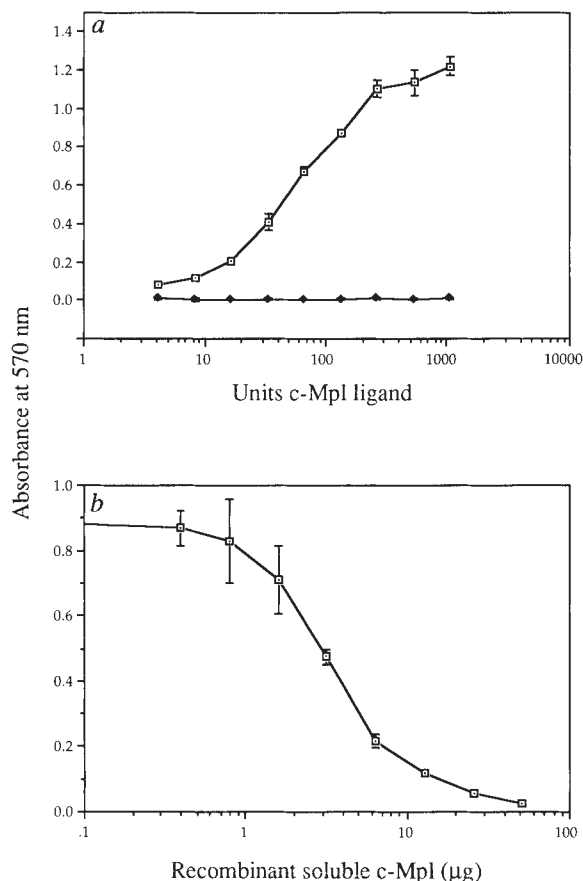
The protein encoded by pZGmpl-1081 has an activity consistent with that of a *c-Mpl* ligand (Fig. 1). Recombinant protein produced in baby hamster kidney (BHK) cells transfected with pZGmpl-1081 supports the proliferation of BaF3/MPLR1.1 cells expressing *c-mpl* but not the proliferation of parental BaF3 cells lacking expression of the receptor. The proliferative activity of the recombinant protein on BaF3/MPLR1.1 cells is dose dependent (Fig. 1a). Activity was not affected by the presence of neutralizing antibodies to interleukin-3 (IL-3) or IL-4, two cytokines found to support the proliferation of both BaF3/MPLR1.1 and BaF3 cells. Specificity of pZGmpl-1081 recombinant protein to *c-Mpl* was further indicated by the depletion of activity on addition of a soluble form of *c-Mpl* (Fig. 1b). The binding affinity for soluble recombinant *c-Mpl* immobilized on sepharose gel also enabled rapid affinity purification of recombinant *c-Mpl* ligand produced in transfected BHK cells. On elution from the affinity column, the purified *c-Mpl* ligand was fully active.

The predicted mature polypeptide has a two domain structure. The N-terminal domain of 152 amino acids is related to erythropoietin (EPO) (Fig. 2b), a feature not unexpected because *c-Mpl* and the EPO receptor have homologous regions. The domain is also weakly similar to murine interferon alpha-8 (Fig. 2b). No statistically significant similarity to entries in the GenBank<sup>9</sup> was found for the C-terminal domain. A recent report of the FLT3/FLK2 ligand showed that one isoform has a hydrophobic C-terminal extension sequence that might be important for membrane association<sup>10</sup>. Whether the *c-Mpl* ligand C-terminal domain functions in a similar manner is not clear because the domain is considerably less hydrophobic, particularly when glycosylated. The junction between the N-terminal EPO-like domain and the C-terminal domain contains a pair of arginine residues resembling a dibasic proteolytic cleavage site<sup>11</sup>. We have, however, no evidence at this time to suggest that recombinant *c-Mpl* ligand produced in BHK cells is cleaved at this site. Mature EPO is processed from a precursor polypeptide by proteolytic cleavage at an arginine residue located at a similar position<sup>12</sup>. Characterization of the cDNA and gene encoding human *c-Mpl* ligand revealed that the human protein also has a two-domain structure similar to that of mouse *c-Mpl* ligand (manuscript in preparation).

† To whom correspondence should be addressed.

**FIG. 1** Proliferative activity of recombinant pZGmpl-1081 protein on c-Mpl ligand-responsive cells and inhibition of activity by soluble c-Mpl. **a**, The proliferative activity of conditioned media from cultured transfected BHK cells expressing pZGmpl-1081 protein was assayed on (□) BaF3/MPLR1.1, an IL-3-dependent cell line, responsive to c-Mpl ligand due to expression of a transfected c-Mpl-encoding cDNA; and (◆) BaF3 cells, an IL-3-dependent cell line lacking expression of *c-mpl*. Cell proliferation was measured by a colorimetric assay as described below. The amount of activity giving rise to 50% of maximal proliferative response is defined as 50 units. **b**, Inhibition of activity by soluble c-Mpl. One hundred units of c-Mpl ligand and the indicated amounts of recombinant soluble c-Mpl were added to  $1 \times 10^5$  BaF3/MPLR1.1 cells. Cell proliferation was assayed after 3 days at 37 °C. Standard deviations are represented by error bars.

**METHODS.** We transfected a murine c-Mpl-encoding cDNA into BaF3 cells to produce a cell line designated as BaF3/MPLR1.1 that is responsive to the proliferative activity of c-Mpl ligand. BaF3 and BaF3/MPLR1.1 cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.0036% 2-mercaptoethanol, and 4% conditioned media from cultured WEHI-3 cells (Collaborative Research) as a source of IL-3. WEHI-3 conditioned medium in the culture medium was omitted when assaying for c-Mpl ligand activity. For the assay, 100- $\mu$ l aliquots of diluted conditioned media from pZGmpl-1081-transfected BHK cells were added to  $1 \times 10^5$  washed BaF3 or BaF3/MPLR1.1 cells and cultured for 3 days at 37 °C. Cell proliferation was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)<sup>21</sup>. Soluble c-Mpl was produced in BHK cells expressing a stably integrated copy of a truncated c-Mpl-encoding cDNA. The cDNA was constructed by deleting sequences encoding the transmembrane and cytoplasmic domains of full-length *c-mpl* cDNA by PCR mutagenesis (manuscript in preparation). A cell line producing elevated levels of c-Mpl ligand was isolated by mutagenesis of BaF3/MPLR1.1 cells in the presence of 0.15% (v/v) EMS<sup>8</sup> followed by selection for mutant clones exhibiting exogenous factor independent autocrine growth. Autocrine mutants resulting from the activation of the endogenous IL-3 or IL-4 genes were identified by use of neutralizing antibodies and were eliminated from the screen. A mutant cell line, 24-11, producing c-Mpl ligand was identified by the ability of its conditioned media specifically to support proliferation of BaF3/MPLR1.1 cells expressing *c-mpl* but not parental BaF3 cells lacking expressed receptor. An expression cDNA library was constructed from 24-11 cell line poly(A)<sup>+</sup>RNA. First-strand cDNA was synthesized using primer 5'-GTGGTGCTCAGCACTACTCGAGGGT<sub>18</sub>, containing a *Xho*I restriction site. Second-strand cDNA was synthesized using a modification of the Gubler and Hoffman protocol<sup>22</sup>. Following the ligation of *Eco*RI adaptors, the cDNA was directionally cloned into the mammalian expression vector pDX<sup>23</sup>, which had been modified to receive *Eco*RI-*Xho*I inserts. The resulting 24-11 expression cDNA library was plated onto 2,000 ampicillin plates at a density of ~250 bacterial colonies



per plate. Pools of plasmid DNA for transfection were prepared from each bacterial plate using MAGIC MINIPREPS DNA purification resin (Promega) and were transfected into BHK 570 cells using LIPOFECTAMINE (Gibco-BRL). Cultured conditioned media from transfected BHK 570 cells were collected after 48 h and assayed for proliferative activity on BaF3/MPLR1.1 and BaF3 cells as described above. Two positive pools, 1081 and 1815, which supported proliferation of BaF3/MPLR1.1 but not BaF3 cells, were successively broken down into smaller plasmid pools until single clones, pZGmpl-1081 and pZGmpl-1815, were obtained.

Northern blot analysis using a full-length *c-mpl* cDNA probe showed two low-abundance hybridizing mRNA species of 1.8 and 5 kilobases (kb) in a variety of murine tissues and organs (Fig. 3). It should be noted that EPO mRNA synthesis is limited to the peritubular cells of the kidney<sup>13</sup>. Similarly, the apparent wide tissue distribution of c-Mpl ligand mRNA may be a consequence of expression in a few specialized cell types present in the tissues examined. The 3' untranslated region of c-Mpl ligand mRNA contains a number of short A-U-rich sequences (Fig. 2). Similar sequences have been implicated in destabilization of a number of cytokine mRNAs<sup>14,15</sup>.

The transcript levels in mutant 24-11 cells are about 50-fold higher than in the mouse tissues examined (data not shown). To determine whether the mutagenic event that gave rise to overexpression in 24-11 cells had altered the c-Mpl ligand coding sequences, we isolated the coding sequence of c-Mpl ligand from mouse kidney mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Sequence analysis of the RT-PCR products showed a coding sequence identical to pZGmpl-1081 cDNA isolated from 24-11 cells.

To begin to elucidate the function of c-Mpl ligand in haematopoiesis, we examined the effect of the recombinant protein in animals. BALB/c mice treated with daily intraperitoneal injections of 50 ng units of purified recombinant c-Mpl ligand produced in BHK cells had a greater than fourfold increase in circulating platelet levels after seven days when compared to control animals receiving control buffer (data not shown). Platelet level increase in representative animals could be visualized by the expansion of the buffy coat in haematocrit tubes (Fig. 4). As the white cell count was essentially unchanged in these animals, the enlarged buffy coat is composed almost entirely of platelets. The treated animals exhibited no other signs of pathology except for a greatly increased number of megakaryocytes in bone marrow and spleen<sup>16</sup>. The magnitude of the observed thrombopoietic response in mice is many fold greater than the modest increase in circulating platelet levels observed following the administration of other cytokines such as IL-3, IL-6, IL-11 or c-Kit ligand<sup>17-20</sup>. These results, along with those in the accompanying paper<sup>16</sup> which examines in greater detail the effect of the recombinant protein in animals as well as *in vitro*, suggest



**FIG. 2 a**, Nucleotide sequence of murine c-Mpl ligand cDNA and deduced amino-acid sequence. Numbering is from the 5' end of the DNA sequence and from the initiation methionine of the amino-acid sequence. The predicted signal peptide is underlined; the bar denotes the site of signal peptide cleavage as determined by N-terminal sequence of purified native protein from 24-11 cells and of recombinant protein produced in BHK 570 cells. An upstream in-frame ATG codon is overlined; translation initiated at this codon would give rise to an additional upstream peptide sequence (MAPGKIQGRGPIQGATSVRLAR) that is unlike signal peptides and atypical of homologous cytokines. A pair of arginine residues marking the junction between the N-terminal EPO-like domain and the C-terminal domain are indicated by asterisks. The stop codon is indicated by a full stop. **b**, One possible multiple sequence alignment of erythropoietins from human (hEPO), ovine (sEPO) and murine (mEPO), murine c-Mpl ligand (mML), murine interferon alpha 8 (mIFNA8) and murine interferon beta (mIFNB) (all sequences were obtained from GenBank<sup>9</sup>). The alignment indicates that the N-terminal domain of murine c-Mpl ligand is related to proteins in structural classes that include the haematopoietic cytokines and interferons. The degree of similarity between murine c-Mpl ligand and the other sequences is weak but significant: murine c-Mpl ligand and mEPO are 21.5% identical (indicated by vertical bars), with an additional 23.5% conservative substitutions (defined as amino-acid replacements associated with non-negative scores in a blosum 62 substitution matrix<sup>24</sup>; blosum 62 scores >2.0 are indicated by .; scores >0.0 but <2.0 are indicated by -). The optimized rdf2 z score for the murine c-Mpl ligand and mEPO alignment is 8.9 (z scores >6 generally indicate genuine similarities<sup>25</sup>). Murine c-Mpl ligand and mIFNA8 are 20.5% identical plus 24.1% conservative substitutions; the optimized rdf2 z score is 6.3. Numbering is from the initiation methionine residues; signal sequence cleavage sites are indicated by a vertical line. Potential N-linked glycosylation sites in c-Mpl ligand are indicated by asterisks. The alpha helices determined for interferon beta<sup>26</sup> and proposed for EPO<sup>27</sup> are underlined and overlined, respectively.

**METHODS.** Both strands of pZGmpl-1081 cDNA were sequenced using AmpliTaq DyeDeoxy Terminator Cycle Sequencing on an Applied Biosys-

**a**

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CCTCGTGCCGGCTCTGAGGCCCTTCTCCACCCGACAGACTCTTGGCCACCTCTCTCCACCCGACTCTGCCAAAGACAGACAGCTCAAGCCGCTCCATGGCCGCCAGGAAG 119
ATTGAGGGGAGAGGCCCATACAGGGAGCCACTTCAGTTAGACACCTGGCCAGATGGAGTGACTGATTGCTCTGGCCGCCATGCTTCTTGCAGTGGAAGCACTAACTCTGTCCAGC 239
M E L T D L L L A A M L L A V A R L T L S S 22
CCGAGTCTCTGCTGTGACCCAGACTCTTAAATAAAGTCTGCTGACTCCCACTCTTCCAGCCGACTGAGTCAGTGTCCGACGTCGACCTTTGTCTATCCCTGTCTGCTG 359
P V A P A C D P P R L L N K L S L L L N S R L S C C P D P D V F Y L P L S I P V L L 62
CCTGCTGTGAGCTTTAGCTGGGAGATGGAACACCCAGACGGAACAGGACAGGACAGGACATTCTAGGGGAGTGTCTCTTACTGAGGAGGATGATGGACAGCAGGACAGTGT 479
P A V D F S L G E W K T Q T E Q S K A Q D I L G A V S L L L E G V M A A R G Q L 102
GAACCTCTCTGCTCTATCCCTCTGGGAGAGCTTTCTGGGAGGTCGCTCTCTTGGGGGCCCTGACAGGCCGCTCTCTGACAGGCCAGGACAGCAGCTCAC 599
E P S C L S S L L G Q L S G Q V R L L L G A L Q G L L G T Q L P L Q G R T T A H 142
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K D P N A L F L S L Q Q L L R G K V R F L L L V E G P T L C V R R T L P T T A V 182
CCAAGCAGTACTTCTCACTCTCACATAAAGTTCACCAACAGGACTCTGGATTGTTGGAGACGAACTCAGTGTACAGCCAGCACTGCTGAGCTTCTGAGCAGGCT 839
P S S T S Q L L T L N K F P N R T S G L L E T N F S V T A R T A G P G L L S R L 222
CAGGGATTCAGAGTCAAGATTCTCTGCTGCTCAATCAACCTCCAGTCCCAATCTCTGGATACCTGAACAGACACAGGACCTGGAATGGAATCATGGCTCTTT 959
Q G F R V K I T P G Q L N Q T S R S P V Q I S G Y L N R T H G P V N G T H G L F 262
GCTGGAACCTCACTTCAGACCTGGAAGCTCAGACATCTGCGCGGAGCTTTCAACAAGGCTCCCTGGCATCAACCTCCAGGGTGAATCTCTCTTCCAAAGCTTGTCTGTAT 1079
A G T S L Q T L E A S D I S P G A F N K G S L A F N L Q G G L P P S P S L A P D 302
GGACACACACCTCTCTCTCTCACTGCTTGGCCACCACTGATCTCCACCCAGCTCCAGCCCTGTTCTGACCTTCCACACCATGCTAATCTACCGCCCTCATCA 1199
G H T P F C P P S P A L P T T H G S P P Q L H P L F P D P S T T M P N S T A P H P 342
GTCACAACTACCTCTCCAGGAATTTGTCTCAGGAACATAGCGGGGACCTGGCCAGTGGCTGTCAGCTTCTCTGGGGACAGCTTCCCCAGGAAGCTGAGAGCAGCTG 1319
V T M Y P H P R N L S G E T . 356
CATCTGCTCCAGATGTTCTGCTCTTCACTAAAGGCCCTGGGAGGGATACACAGCAGCTGGAGATGTAATAATTTAGGAGCTATTTTTTAACTATCAGCAATATTCATCAGAG 1439
AGCTAGCATCTTTGTCTATTTTCGGTATAAATTTGAAATCACTA 1486

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**b**

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hEPO  MGVECPAWLWLLLSLPLGLPVLG  APPRLICDSRVLYRYLEAKEAENITTCGAECNSLNIITVPDKVNFYAWKRMEVG--QQAVEVMQ  92
sEPO  MGARDCTPLLLLLLSLPLGLPVLG  APPRLICDSRVLYRYLEAREANATMGCAEGCSFSENIITVPDKVNFYAWKRMEVG--QQALEVMQ  92
mEPO  MGVERPPTLL-LLLSLPLGLPVLG  APPRLICDSRVLYRYLEAKEAENITMGCAEGPRSENIITVPDKVNFYAWKRMEVE--EQALEVMQ  91
mML   MELTDLALLAAMLLAVARLTLS  SPVAPACDPRLLNKLRLDSSLHSRLSGCPDVPDPLSIPVLLPAVDFSLGKWTQTEQSKAGDILGAV  88
mIFNA8  MARLCAFLMVLAVLSYWPCTSLG  -----CDLPQTHNLRNKRALTLLVKMRRLS--PLSCLDRKDFGFPQEKVGAQIQ-EQAQIPVLT  81
mIFNB  MNWRILHAAFLLCSTTALS  -----INYLQQLQERTNIRKQELLEQLN--GKINLYTRADFIPMEM--TEKMQ-KSYTAFAIQ  77

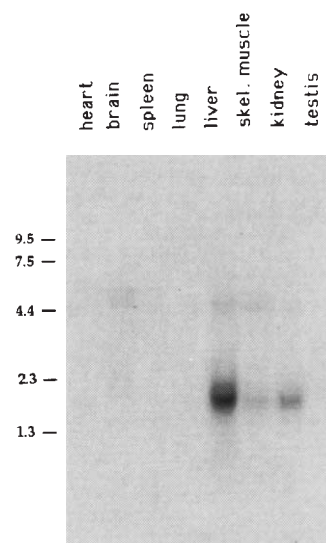
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sEPO  GLALLSEAFRGQALLANASQPCALRLHVDKAVSGRLSTLLRA-LGAQKEAIPDPATPSAAPLRIFTVDALSKLFRVYNSFLRGKLYTG  186
mEPO  GLSLLSEALQAQALLANSOPPETLQLHIDKAISSRLSTLLRV-LGAQKELMSPPDTP-PAPLRLTVDFTCKLFRVYANFLRGKLYTG  184
mML   SLLLEGVMAARGGLEPSCLS--LLGQLSGQVRLLLGALQGLLTGTLPLQGRTHAHKD-----PNALFLSLQQLRGKVRFL  165
mIFNA8  ELTQQLIALFTSKDSSAAWNA--TLLDSFCDNLHQLNDLQGLMQQVEIQALPLTQED-----SLLAVRYFHRITVFLREKHSPCA  163
mIFNB  EMLQNVFLVFRNNSSTGWNE--TIVVRLDELHQQTFLKTVLEE-KQERLTWEMSS-----TALHLKSYRVORYLKLKMYNSA  158

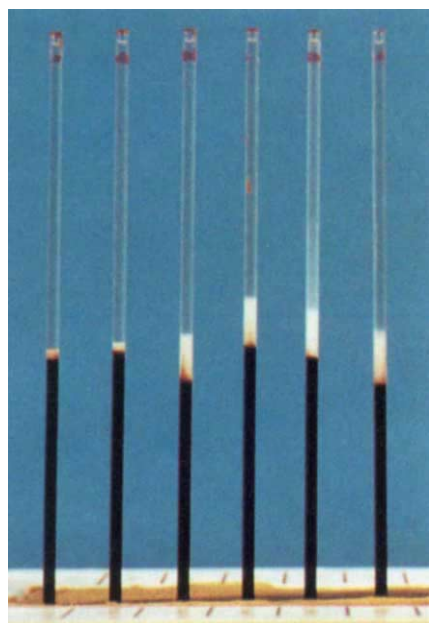
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sEPO  -----EACRRGDR  194
mEPO  -----EVCRRGDR  192
mML   VEGPTLCVRRTLPPTVAPSSQSLTLNKFNPRTSGLLETNFSVTARTAGPGLLSRLQGRVKITPGQLNQTSSRPVQISGYLNRTHPVNGTHG  260
mIFNA8  WEVVRAEVWRALSSAKLLARLNDE  189
mIFNB  WMVVRAEIFRNFLIRRLTRNFQ  182
mML   LFAGTSQTLASDISPAGFNKGLAFNLQGLLPSPSLAPDGHPTFPSPALPTTHGSPQLHPLFDPSTTMPNSTAPHPVTMYPHNRSLQET  356

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tems model 373A sequencer. c-Mpl ligand for N-terminal amino-acid sequence determination was purified by affinity chromatography using immobilized soluble recombinant c-Mpl and was sequenced using an Applied Biosystems model 476A sequencer.

**FIG. 3** Northern blot hybridization of murine RNA with c-mpl cDNA probe. **METHODS.** A murine multiple tissue Northern blot (Clontech Laboratories) was hybridized with a probe generated by random priming of full-length murine c-mpl cDNA in RAPID HYBE solution from Amersham as per the manufacturer's instructions. Each lane contains about 2 µg of poly(A)<sup>+</sup> selected mRNA. The blot was washed in 0.2 × SSC, 0.2% SDS at 65 °C and exposed to a phosphor plate for 3 days.





Control mice      c-Mpl ligand-treated mice

FIG. 4 Haematocrit of peripheral blood from representative control mice and mice treated with seven daily intraperitoneal injections of recombinant c-Mpl ligand. The increase in the size of the buffy coat of the treated animals is due to increases in platelets.

**METHODS.** BALB/c mice (Simonsen Labs) were treated with seven daily intraperitoneal injections of either 50 ng of recombinant c-Mpl ligand or control buffer. On day 7, animals were anaesthetized and samples collected by an orbital eye bleed. Blood was drawn directly into heparinized Micro-Hematocrit capillary tubes (Oxford Labware) and spun for 5 min in a Readacrit Centrifuge (Clay Adams).

strongly that the c-Mpl ligand is thrombopoietin, the long-sought major physiological regulator of megakaryocytopoiesis and platelet production. □

## Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin

Kenneth Kaushansky\*, SI Lok†, Richard D. Holly†, Virginia C. Broudy\*, Nancy Lin\*, Mason C. Bailey†, John W. Forstrom†, Michele M. Buddle†, Pieter J. Oort†, Fredrick S. Hagen†, Gerald J. Roth\*, Thalia Papayannopoulou\* & Donald C. Foster†

\* Division of Hematology, University of Washington School of Medicine, Seattle, Washington 98195, USA

† ZymoGenetics Corporation, 4225 Roosevelt Way NE, Seattle, Washington 98105, USA

THE development of blood cells including expansion of megakaryocyte progenitor cells requires the interplay of marrow stromal cells and polypeptide cytokines<sup>1-10</sup>. Recently, characterization of c-Mpl, the receptor encoded by the proto-oncogene *c-mpl*, revealed structural homology with the haematopoietic cytokine receptor family<sup>11</sup>, and its involvement in megakaryocyte development<sup>12</sup>. We report here that the ligand for c-Mpl<sup>13</sup> is relatively lineage specific, works both alone and synergistically with early acting cytokines to support megakaryocyte colony formation, and acts at a late stage of development to increase megakaryocyte size, polyploidization and expression of differentiation markers. *In vivo*, c-Mpl ligand stimulates platelet production by greatly expanding marrow and splenic megakaryocytes and their progenitors, and by shifting the distribution of megakaryocyte ploidy to higher values. Thus, as c-Mpl ligand has the expected characteristics of the major regulator of megakaryocyte development, we propose that it be termed thrombopoietin.

Complementary DNA encoding murine c-Mpl ligand was subcloned into pZEM229R<sup>14</sup> and used to direct BHK cell production of the recombinant protein. c-Mpl ligand alone could support the formation of megakaryocyte-containing colonies (Table 1). However, individual colonies were small, suggesting that the target cells were late, committed progenitors. In contrast, cultures that also contained interleukin-3 (IL-3) or c-Kit ligand (KL), proteins previously shown to affect megakaryocytic proliferation<sup>9-10,15</sup>, produced supra-additive numbers of very large colonies. Despite these effects on megakaryopoiesis, c-Mpl ligand only minimally affected erythroid and granulocyte-macrophage colony formation. To determine the timing of cytokine action, sequential addition experiments were done. Delayed addition of c-Mpl ligand failed to alter the quantity or quality of the resultant megakaryocyte colonies. In contrast, delayed addition of IL-3 or KL substantially reduced colony number (Table 1) and size. Thus, IL-3 and KL appeared to act at an early stage and c-Mpl ligand at a late stage of megakaryocyte development. Similar conclusions were derived from sequential addition studies using a soluble c-Mpl receptor that neutralized the biological activity of the ligand.

To determine the effects of cytokine combinations on the size, number and ploidy of developing megakaryocytes, marrow cells were purged of mature megakaryocytes and grown in serum-free suspension culture for six days. Compared to cells from control cultures, c-Mpl ligand-induced megakaryocytes were more numerous, larger, had a greater nuclear mass with more basophilic cytoplasm, and were frequently seen to be blebbing off platelets (Fig. 1a-c). The mean diameter of acetylcholinesterase (AChE)-stained megakaryocytes was  $30 \pm 0.2 \mu\text{m}$  in control ( $n = 58$ ),  $43 \pm 2.0 \mu\text{m}$  in IL-3 ( $n = 71$ ),  $58 \pm 2.0 \mu\text{m}$  ( $n = 138$ ) in c-Mpl ligand and  $53 \pm 2.4 \mu\text{m}$  ( $n = 113$ ) in c-Mpl ligand plus IL-3-con-

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taining cultures. A quantitative fluorometric assay of AChE activity was used to determine megakaryocyte mass. Cytokine combinations containing c-Mpl ligand resulted in greater total AChE activity (Fig. 1d). And by flow cytometric analysis we found that culture in c-Mpl ligand resulted in a significant shift to more highly polyploid megakaryocytes (Fig. 1e).

To determine if c-Mpl ligand induced expression of megakaryocytic surface markers, we tested its effects on human cell lines that express *c-mpl*<sup>12</sup> and can undergo megakaryocytic differentiation<sup>16</sup>. Cell lines MO7e and KU812 but not HEL or CMK cells proliferate in response to murine c-Mpl ligand (Fig. 2a) indicating that the murine protein could engage the human receptor. Using fluorescence cell sorting we found that the cellular display of GP Ib, a platelet membrane differentiation antigen, increased in CMK cultures containing c-Mpl ligand (Fig. 2b). This result indicates that c-Mpl ligand directly induces a differentiation programme in cells committed to the megakaryocytic lineage.

To examine the effects of c-Mpl ligand *in vivo*, culture medium from BHK cells engineered to produce the protein was purified and injected into mice. The results from the first experiment are

presented in the accompanying paper<sup>13</sup>. To confirm and expand these results, purified protein or vehicle alone was injected daily into groups of five mice. After a three day lag period the platelet counts, but not the haematocrit or white blood cell counts, began to rise, and by the fifth day of treatment the platelet counts were elevated a mean of fourfold over that of the vehicle-treated animals. Essentially identical results were obtained in three additional experiments. These results compare favourably with the reported 30–70% increase in platelet count following the high-dose administration of IL-3, IL-6, IL-11 or KL to mice or primates<sup>17–21</sup>. Histological examination of the marrow (Fig. 3a, b) and spleen of these animals confirmed that the rise in platelet count was due to increased production of very large megakaryocytes.

To assess the effects of c-Mpl ligand on haematopoietic progenitor cells, the total femoral and splenic CFU-Mk, CFU-GM, BFU-E and CFU-E were determined. About 100 ng per day of highly purified c-Mpl ligand lead to a greater than 20-fold increase in CFU-Mk in both tissues (Fig. 3c–e). Only minimal effects were noted on the erythroid and granulocytic progenitor populations (data not shown). In contrast, the administration

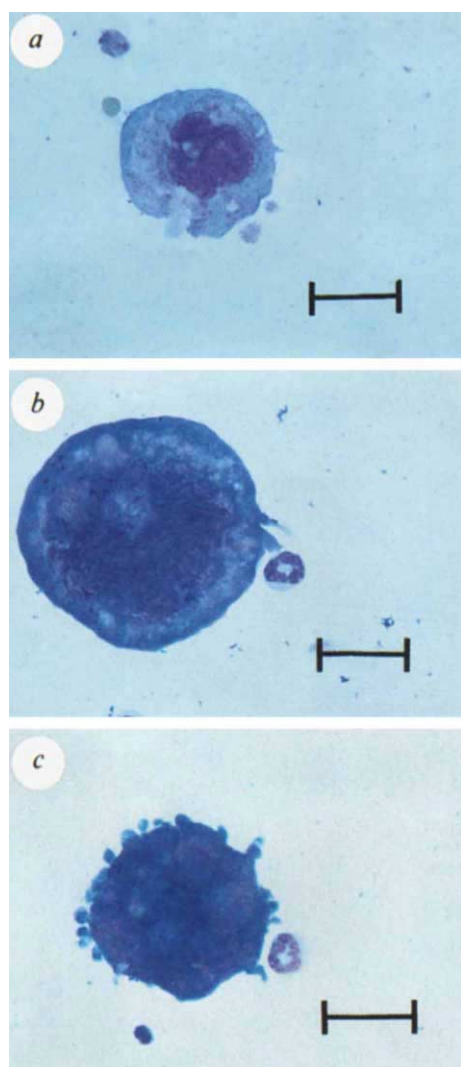
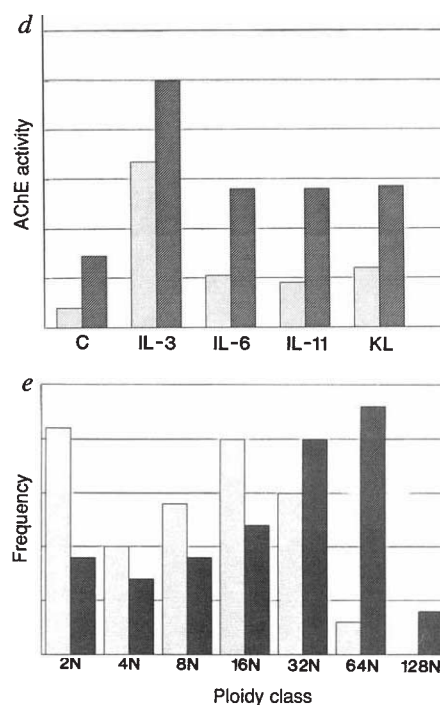


FIG. 1 Characteristics of megakaryocytes grown in the presence of c-Mpl ligand. Normal murine marrow cells were obtained by flushing marrow cavities with IMDM supplemented with 10% fetal calf serum (FCS) through 20- and 25-gauge needles. The resultant cell populations



were nearly devoid of AChE-positive cells, thereby assuring that the characteristics of cultured megakaryocytes were entirely dependent on *in vitro* culture conditions. Cells were washed to eliminate FCS and were cultured ( $1 \times 10^6$  per ml) for six days in IMDM supplemented with 1% Nutridoma with or without purified cytokines at levels selected for optimal synergistic activity (150 U c-Mpl ligand, 1.5 ng IL-3, 15 ng IL-6, 5 ng IL-11 or 2 ng KL). Individual cellular morphology was determined by staining cytopreparations with AChE and Giemsa for cultures grown in IL-3 (a) or c-Mpl ligand (b, c). Scale bars, 20  $\mu$ m. d, A quantitative fluorescent assay of AChE activity was used to determine megakaryocyte mass<sup>6</sup>. The white bars represent the indicated cytokine alone; the hatched bars, the results using the indicated cytokine(s) plus c-Mpl ligand. The data represent the mean of triplicate determinations; essentially identical results were obtained in two additional experiments. e, Two-colour FACS was conducted using the megakaryocyte-specific rat mAb 4A5 (ref. 25) and propidium iodide as a measure of cellular DNA content<sup>26</sup>. The data represent the fraction of megakaryocytes (4A5-positive cells) containing the displayed ploidy value (number  $\times$  haploid genome) for IL-11- (white bars) and c-Mpl ligand (hatched bars)-stimulated cultures.

of 10  $\mu\text{g}$  IL-6 or 3  $\mu\text{g}$  IL-11 per day to mice for 5–7 days increases marrow or splenic CFU-Mk a maximum of threefold<sup>17,19,20</sup>. As these late progenitor cells are not thought to self-renew, our results strongly suggest that in addition to its proliferative effects on CFU-Mk and its effects to induce differentiation (polyploidization) of mature megakaryocytes, c-Mpl ligand also works on

very early haematopoietic cells to expand the pool of committed megakaryocytic progenitors. Previous studies have suggested that a hierarchical system accounts for haematopoiesis in which the survival and proliferation of progenitor cells is supported by multiple early acting growth factors, and their differentiation to mature blood cells supported by a single, lineage-specific late-

FIG. 2 The growth and differentiation of human megakaryocytic cell lines. *a*, KU 812-F cells ( $3 \times 10^5$  per ml) were cultured for 19 days in RPMI supplemented with 10% FCS, antibiotics and either 300  $\text{U ml}^{-1}$  c-Mpl ligand as conditioned culture medium, 5  $\text{U ml}^{-1}$  EPO, both or with no additional stimulus. The fold increase in cell number over cells grown without growth factor supplementation is shown as a function of time for c-Mpl ligand (---), EPO (....) or c-Mpl ligand plus EPO (—)-treated cultures. *b*, CMK cells were grown as described above for KU 812-F. After 11 days culture, cells were sorted using a polyvalent antiserum which recognizes human GP Ib<sup>27</sup>, a component of the platelet von Willibrand factor receptor.

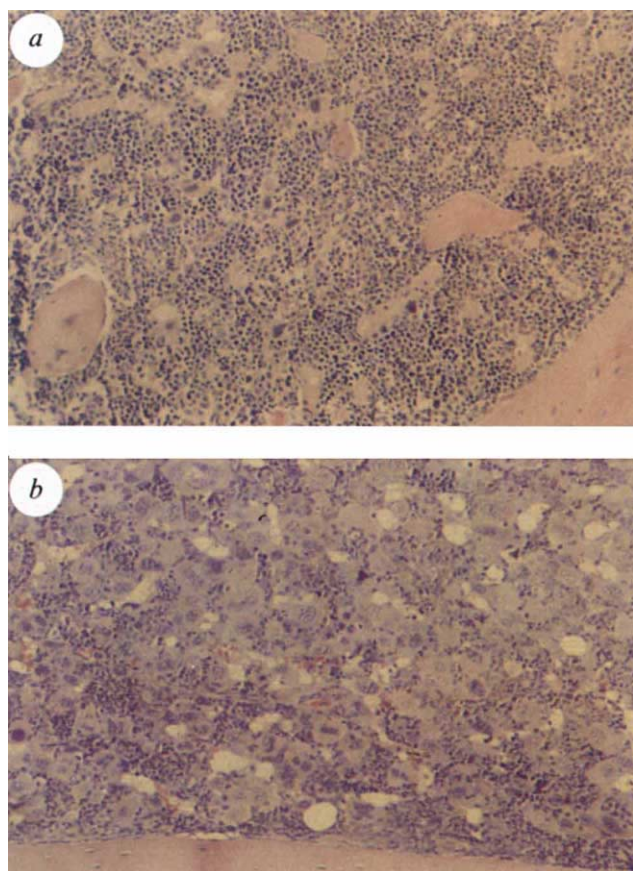
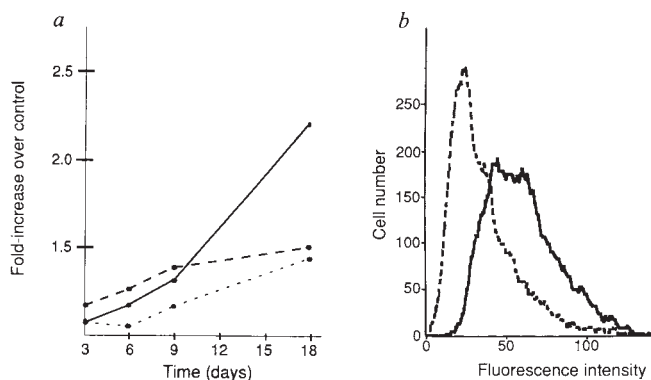
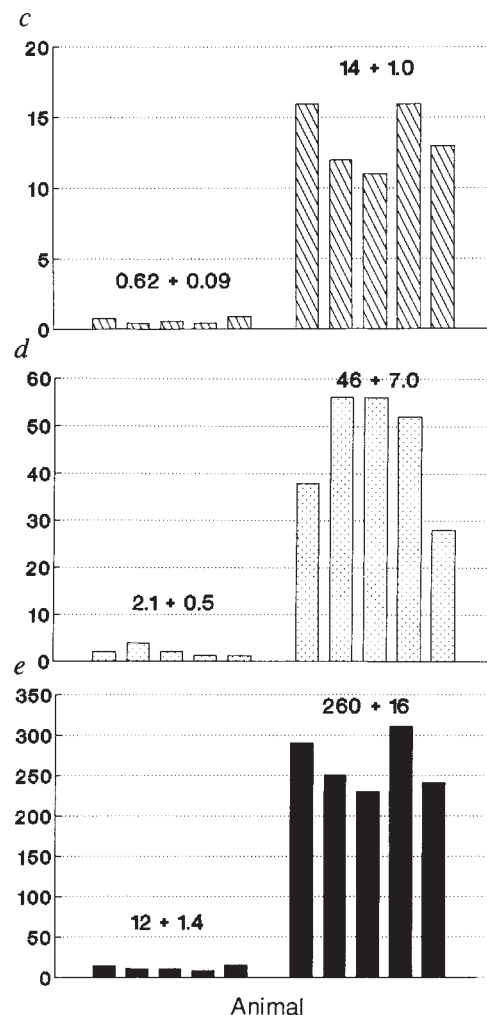


FIG. 3 *In vivo* activity of the c-Mpl ligand. Groups of five BALB/c mice were injected intraperitoneally daily for 5 days with either 100 ng highly purified c-Mpl ligand or vehicle alone. The haematocrit and white blood cell counts of the c-Mpl ligand-treated animals were unchanged but the platelet counts rose a mean of fourfold over that of the control animals. The spleen and femurs of each of the animals were obtained, examined histologically and single-cell suspensions prepared. Femoral sections ( $\times 100$ ) of a control (*a*) and a c-Mpl ligand-treated (*b*) mouse are shown. Single-cell suspensions of flushed marrow or minced spleen were used to determine the total number of megakaryocytic (CFU-Mk), early erythroid (BFU-E), late erythroid (CFU-E) and myeloid (CFU-GM) progeni-



tors by semi-solid culture in the presence of optimal concentrations of cytokines (IL-3, IL-11 and Mpl ligand for CFU-Mk, IL-3 for CFU-GM, PWM-SCM and EPO for BFU-E and EPO alone for CFU-E). The data represent the megakaryocyte progenitor number ( $\times 10^{-3}$ ) for each femur (*c*), spleen (*d*) and whole animal (*e*; calculated as described<sup>28</sup>). The mean values  $\pm$  s.e.m. are displayed above each set of results.



TABLE 1 Haematopoietic colony formation

|                | CFU-Mk    |          |         | CFU-GM   |           | BFU-E    |          | CFU-E |          |
|----------------|-----------|----------|---------|----------|-----------|----------|----------|-------|----------|
|                | Alone     | + ML     | + d3ML  | Alone    | + ML      | Alone    | + ML     | Alone | + ML     |
| Control        | 0 ± 0     | 37 ± 8.0 |         | 0 ± 0    | 1.8 ± 1.8 | 0 ± 0    | 0 ± 0    | 0 ± 0 | 0 ± 0    |
| IL-3           | 30 ± 6.0  | (100)    | 92 ± 13 | (100)    | 117 ± 7.0 |          |          |       |          |
| KL             | 11 ± 3.0  | 110 ± 31 |         | 49 ± 2.4 | 42 ± 4.4  |          |          |       |          |
| IL-6           | 1.0 ± 2.0 | 38 ± 6.0 |         |          |           |          |          |       |          |
| IL-11          | 2.0 ± 2.0 | 31 ± 6.0 |         |          |           |          |          |       |          |
| EPO            |           |          |         |          |           | 15 ± 3.5 | 13 ± 4.3 | (100) | 110 ± 10 |
| PWM-SCM        | 56 ± 4.0  |          |         |          |           | 0 ± 0    | 0 ± 0    |       |          |
| PWM-SCM + hEPO |           |          |         |          |           | (100)    | 101 ± 15 |       |          |

Marrow cells ( $8 \times 10^4$ ) from BDF<sub>1</sub> mice, obtained by femoral aspiration, were plated in 1-ml cultures containing Iscove's modified Dulbecco's medium (IMDM), 15% horse serum, antibiotics, and combinations of 150 U murine c-Mpl ligand (ML), 180 U murine IL-3, 5 ng human IL-6, 5 ng human IL-11 or 20 ng murine KL and then made semi-solid with 0.275% agar. In some experiments c-Mpl ligand addition was delayed until day 3 of culture. Fifty units of c-Mpl ligand or IL-3 activity was defined as the dilution that produced half-maximal activity in a MTT assay based on IL-3-dependent BaF3 cells expressing murine c-Mpl. The cultures were incubated at 37 °C in a fully humidified atmosphere containing 5% CO<sub>2</sub>. After 5–6 days, colonies derived from megakaryocytic progenitors (CFU-Mk) were enumerated by microscopic examination of whole cultures. The megakaryocytic nature of these colonies was verified using AChE staining. Essentially identical results were obtained in a limited number of studies using 50 pg ml<sup>-1</sup> highly purified c-Mpl ligand alone and in combination with IL-3. Early erythroid progenitors (BFU-E) were cultured in IMDM supplemented with 30% fetal calf serum, 1% bovine serum albumin,  $5 \times 10^{-5}$  β-mercaptoethanol and made semi-solid with 1.4% methylcellulose. Cytokines used included 2 U human erythropoietin (EPO), 1.5% pokeweed mitogen-stimulated murine spleen cell conditioned medium (PWM-SCM) and 150 U ml<sup>-1</sup> c-Mpl ligand. Late erythroid progenitors were quantified in a plasma clot assay as previously described<sup>24</sup>. Megakaryocyte colonies contained greater than 3 polyploid cells, myeloid and erythroid colonies contained >40 cells. The results have been indexed to that of the maximal colony growth and represent the mean (±s.e.m.) of at least three separate experiments of two to three replicate plates. In a typical experiment, IL-3 plus c-Mpl ligand resulted in 18 CFU-Mk-, 140 CFU-GM- and 25 BFU-E-derived colonies per  $8 \times 10^4$  cells plated; EPO alone typically resulted in 120 CFU-E per  $3 \times 10^4$  cells plated in plasma clot assays.

acting cytokine<sup>4,5</sup>. Although evidence in support of this hypothesis is available for the erythroid and granulocytic systems, our results force a reconsideration of the existence of distinct divisions between early and late-acting growth factors<sup>17,22</sup> for the megakaryocytic lineage.

In summary, c-Mpl ligand acts to stimulate haematopoietic progenitors to proliferate and differentiate into megakaryocytes and platelets. Our data suggest that this process may be initiated by IL-3 or KL and completed by c-Mpl ligand, which induces endomitosis, cytoplasmic expansion, membrane maturation and platelet release. The relative lineage specificity and range of target-cell effects of c-Mpl ligand corresponds to the effects of erythropoietin on CFU-E and red cell development<sup>23</sup>, and by analogy suggests that the c-Mpl ligand should be termed thrombopoietin. □

## c-Mpl ligand is a humoral regulator of megakaryocytopoiesis

Françoise Wendling\*, Eugene Maraskovsky†, Najet Debili\*, Christina Florindo†, Mark Teepe†, Monique Titeux\*, Nassia Methia\*, Janine Breton-Gorius‡, David Cosman† & William Vainchenker\*

\* INSERM U362, Institut Gustave Roussy, 94805 Villejuif, France

† Immunex Corporation, 51 University Street, Seattle, Washington 98101, USA

‡ INSERM U91, Hôpital Henri Mondor, 94010 Créteil, France

MEGAKARYOCYTOPOIESIS is the cellular developmental process that leads to platelet production. At least two humoral growth factors may be necessary for megakaryocyte proliferation and maturation. One is a megakaryocyte-colony stimulating factor (MK-CSF) which induces the proliferation and differentiation of megakaryocyte progenitors<sup>1,2</sup>, and the second, thrombopoietin, is a megakaryocyte maturation factor<sup>3</sup>. Neither of these factors has been fully characterized. The proto-oncogene *c-mpl*, an orphan member of the haematopoietin receptor family<sup>4–6</sup>, is specifically involved in megakaryocyte regulation<sup>7</sup>. Here we present evidence that the *c-mpl*-encoded receptor binds a ligand (c-Mpl ligand) which is a humoral factor implicated in platelet homeostasis. Our results suggest that c-Mpl ligand, thrombopoietin and MK-CSF might be the same molecule.

To determine whether the c-Mpl receptor (Mpl-R) could bind a ligand and transmit a mitogenic signal, we used the factor-dependent BAF/BO3 cells<sup>8</sup> transfected with the full-length murine *c-mpl* complementary DNA<sup>6</sup> (BAF/*mpl*) and a soluble fusion protein consisting of the extracellular domain of the murine Mpl-R coupled to the Fc fragment of a human IgG1 (mu-Mpl/Fc fusion protein). As an attempt to identify a source of Mpl-R ligand, we induced a profound cytopenia in mice by irradiation. There was no incorporation of tritiated thymidine

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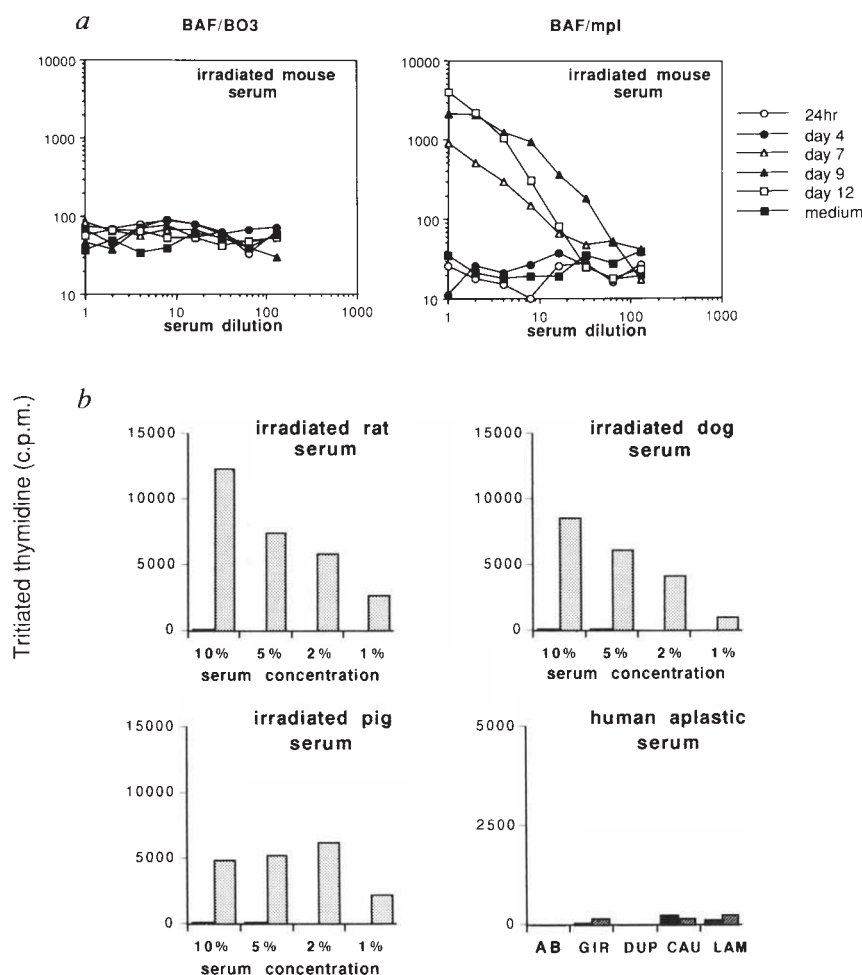
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**FIG. 1** Induction of proliferation of the parental BAF/BO3 and the transfected BAF/*mpl* cells with sera from sublethally (7 Gy) irradiated mammals. **a**, Kinetics of detection of Mpl-L in sera from total body post-irradiated C57BL/6 mice. Sera were collected at times indicated and assayed by titration over twofold dilutions. Thymidine incorporation is presented as mean c.p.m. for triplicate wells. **b**, Wistar rats, beagle dogs and a pig were totally irradiated and their sera were collected on day 9 post-irradiation. Human serum from 4 patients totally irradiated for bone marrow transplantation regimen was collected when the peripheral platelet number was below  $3 \times 10^9 \text{ l}^{-1}$ . Serum from normal individuals (AB) was used as a control. Thymidine incorporation is presented as mean c.p.m. of triplicate wells minus background incorporation. Black bars, BAF/BO3; grey bars, BAF/*mpl*.

**METHODS.** To create the BAF/*mpl* cell line, the full-length murine *c-mpl* cDNA<sup>6</sup> was modified by the replacement of the *c-mpl* signal peptide by that of the murine IL-7<sup>17</sup> followed by the Flag epitope tag sequence<sup>18</sup>. The Flag murine *c-mpl* cDNA was cloned into the mammalian expression vector pDC206<sup>19</sup> and electroporated into BAF/BO3 cells together with pSV2neo. Conditions for growth, electroporation and G418 selection of BAF/BO3 cells have been described<sup>6,20</sup>. After selection in G418 and 1% WEHI-3B conditioned medium, three rounds of fluorescence-activated cell sorting were done by indirect staining with monoclonal mouse anti-Flag antibodies<sup>18,21</sup>. This gave >90% of the cells that demonstrated specific surface staining and remained stable after continuous passage. BAF/*mpl* cells continued to require IL-3 for growth and dose-response curves paralleled those of BAF/BO3. Exponentially growing BAF/BO3 and BAF/*mpl* cells were starved of IL-3 for 20 h before being seeded ( $2.5 \times 10^4$  cells per 100  $\mu\text{l}$ ) in 96-well plates. After 24-h culture, 0.5  $\mu\text{Ci}$  of 6-<sup>3</sup>H thymidine (specific activity = 185 GBq  $\text{mmol}^{-1}$ ) was added. Cultures were collected onto glass fibre filters and radioactivity incorporated into cellular DNA was determined by liquid scintillation counting.

above background levels on the parental BAF/BO3 cells (Fig. 1a). In contrast, sera from post-irradiated mice induced high incorporation of tritiated thymidine into BAF/*mpl* cells. The BAF/*mpl* proliferative activity(ies) was detectable by day 7 after irradiation, with a peak titre at day 9 which remained detectable on day 12. This activity(ies) was dose-dependent and induced BAF/*mpl* proliferation even at a 1:100 dilution. We then investigated whether the murine Mpl-R could be stimulated by activities in sera from other mammals subjected to irradiation. Sera from post-irradiated rats, dogs and pigs also dramatically induced BAF/*mpl* proliferation in a dose-dependent manner, whereas none of these sera induced BAF/BO3 proliferation. In contrast, BAF/*mpl* cells did not respond to stimulation with human aplastic sera<sup>9,10</sup>, suggesting a species specificity (Fig. 1b).

To demonstrate that induction of BAF/*mpl* proliferation correlated with the presence of an Mpl-R specific ligand, sera from irradiated mammals were adsorbed on the mu-Mpl/Fc or the control mu4-1BB/Fc fusion proteins. Results show that the activity inducing BAF/*mpl* proliferation was totally removed after adsorption on the mu-Mpl/Fc protein (Fig. 2). In contrast, adsorption on the mu4-1BB/Fc protein only slightly decreased the proliferative capability of these sera. Thus, the Mpl-R is activated by a specific ligand (Mpl-L) present in sera from animals made cytopenic by irradiation and corresponds to a receptor chain able to bind a ligand and transduce a signal<sup>6</sup>.



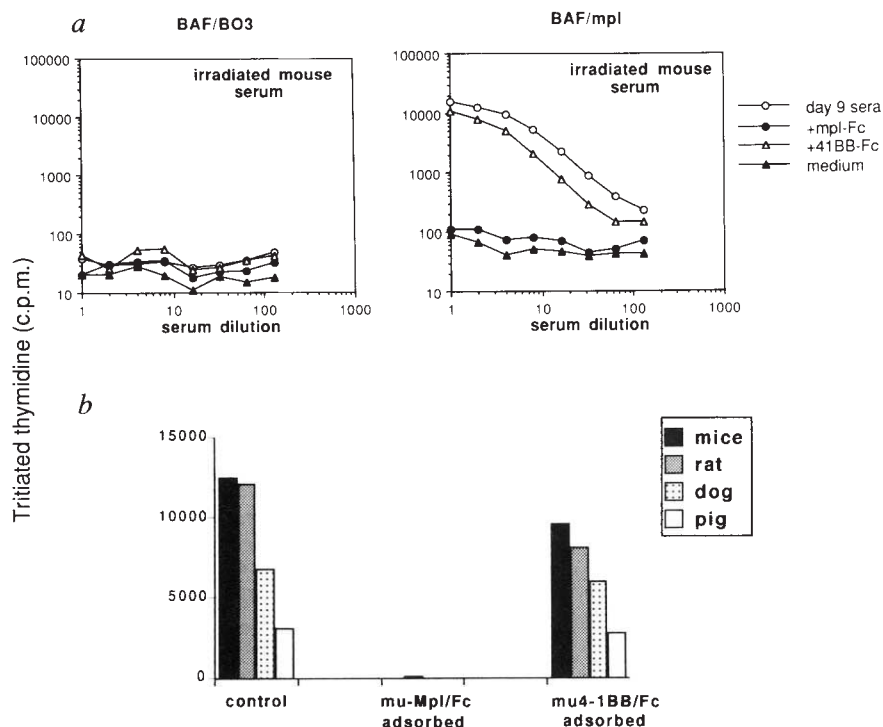
To test whether Mpl-L was a humoral factor whose production was influenced by peripheral platelet counts, we injected mice with a rabbit antimouse platelet antiserum (RAMPS)<sup>11</sup>. A single injection of RAMPS led to a rapid destruction of platelets, with a nadir at 8 h after treatment (Fig. 3a). Sera from 24-h RAMPS-treated mice maximally stimulated BAF/*mpl* proliferation. The activity was detectable 8 h after treatment and remained for 4 days. In contrast, a very low level of BAF/BO3 proliferation was observed at early times indicating that RAMPS treatment induced synthesis of other cytokines (Fig. 3b). To investigate the specificity of this functional activity, sera from 24-h RAMPS-treated mice were adsorbed on both receptor fusion proteins. When adsorbed on mu-Mpl/Fc, the proliferative activity was drastically decreased whereas it was only slightly reduced after adsorption on the mu4-1BB/Fc protein (Fig. 3c). Because thrombopoietin<sup>3</sup> (TPO) is defined as a humoral factor rapidly produced after *in vivo* induction of an acute thrombocytopenia, these results strongly suggest that Mpl-L and TPO could be the same molecule.

We next studied whether Mpl-L in irradiated pig serum played a role in regulating the growth and maturation of murine megakaryocytes (MK). From 8 experiments, an average of  $21 \pm 6$  colony forming unit (CFU)-MK-derived colonies per  $5 \times 10^4$  marrow cells was detected in agar semi-solid cultures stimulated with 6.6% pokeweed mitogen-spleen cell conditioned medium (PWM-SCM)<sup>12</sup>. When fetal calf serum (FCS) and PWM-SCM



FIG. 2 Adsorption of Mpl-L in sera from irradiated mammals using soluble receptor-immunoglobulin fusion proteins. *a*, Dose-response curves to sera from day 9 post-irradiated mice before and after adsorption on either the specific mu-Mpl/Fc or the non-relevant mu4-1BB/Fc fusion proteins. *b*, Sera from irradiated mammals were adsorbed on both fusion proteins before being tested for induction of proliferation on the BAF/*mpl* cells at a final concentration of 10%. Data are presented as the mean c.p.m. of triplicate wells.

**METHODS.** To produce the mu-Mpl/Fc fusion protein, the cDNA encoding the extracellular domain of murine *c-mpl* from amino acids 1-480 was fused in-frame to a cDNA for human IgG1-Fc, essentially as described<sup>23</sup>. The fusion protein-encoding insert was assembled in the pBluescript vector and transferred to the mammalian expression vector pDC406<sup>22</sup>. The mu-Mpl/Fc fusion protein was produced by transfection of CV-1/EBNA cells and protein A affinity purification as previously described<sup>23</sup>. The production of the mu4-1BB/Fc fusion protein has been described<sup>19</sup>. Serum (1 ml) was incubated at 4 °C for 45 min with 0.2 µg purified mu-Mpl/Fc or 4-1BB/Fc fusion proteins. This was adsorbed on 100 µl of packed protein A-sepharose beads at 4 °C for 45 min. Medium was separated from beads by centrifugation (5 min, 10,000 r.p.m.) and used directly in the assays. Control serum was treated in the same conditions except that no fusion protein was added.



were replaced with 10% irradiated pig serum, an average of  $15 \pm 6$  MK colonies containing polyploid MK developed. Serum adsorption on the control mu4-1BB/Fc protein only slightly reduced MK colony numbers ( $10 \pm 3$ ). In contrast, mu-Mpl/Fc-adsorbed serum did not induce MK colony formation ( $0.2 \pm 0.5$ ) whereas CFU-GM growth was not impaired (data not shown). The effect of Mpl-L on MK maturation was examined on bone marrow cells grown in liquid cultures for 3 days with FCS and interleukin-3 (IL-3) and then replated for 4 days with either 10% untreated or 10% mu-Mpl/Fc pre-adsorbed pig serum. Cells were immunolabelled with an anti-CD61 monoclonal antibody, stained with propidium iodide and analysed by flow cytometry. MK with ploidy levels of  $32N$  and  $64N$  were detected in cultures containing untreated pig serum, whereas no MK above  $16N$  were seen in cultures with pre-adsorbed serum (data not shown). These results indicate that Mpl-L present in post-irradiated pig serum appears to function as both a MK-CSF (proliferation) and a TPO-like factor (polyploidization).

As shown above, BAF/*mpl* cells expressing the murine Mpl-R were unable to respond to human aplastic sera<sup>9,10</sup>. Because sera from irradiated dogs contain a MK-CSF acting on human marrow<sup>13</sup>, we investigated whether the Mpl-L in pig serum would promote human CFU-MK colony formation. The number of CFU-MK-derived colonies produced in cultures stimulated with either aplastic human or irradiated pig sera was similar. (Fig. 4). When these sera were adsorbed on the mu-Mpl/Fc protein before culture, a large decrease in MK-colony number was observed with the pig serum, whereas only a slight reduction in MK-colony formation was found with the human aplastic serum, and no significant effect was detected with either sera pre-adsorbed on mu4-1BB/Fc. In addition, the absolute number of MK in cultures with untreated and mu4-1BB/Fc-adsorbed pig serum was at least 4 times higher than with a human aplastic serum, and MK number was drastically reduced with mu-Mpl/Fc-adsorbed pig serum (Fig. 4b). To examine directly the ability of Mpl-L in pig serum to induce human MK maturation, CD34-positive cells were purified from bone marrow<sup>7</sup> and grown for 10 days in liquid cultures either with untreated or mu-Mpl/Fc pre-adsorbed irradiated pig serum. Cells were labelled with an

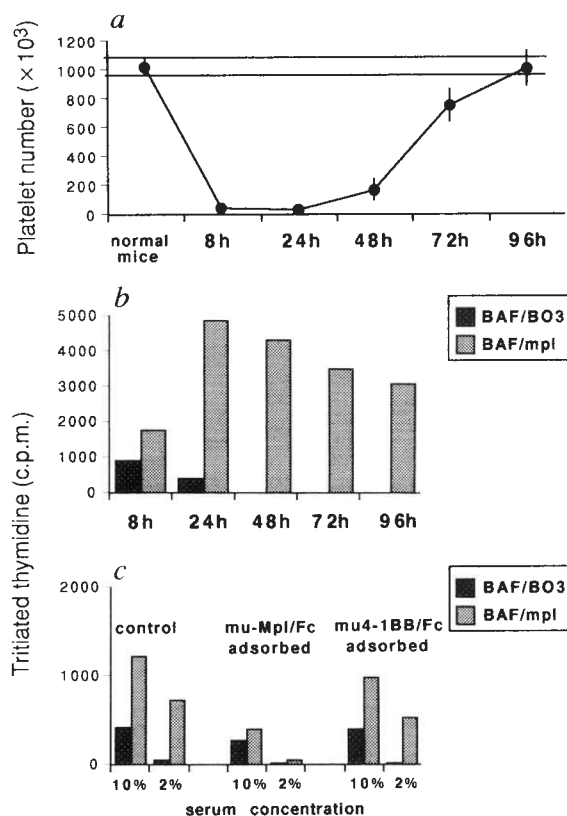


FIG. 3 Induction of Mpl-L in the serum of rabbit anti-mouse platelet polyclonal antibody (RAMPS)-treated mice. *a*, Number of platelets in blood from mice injected intraperitoneally with 0.1 ml RAMPS. Results are mean number of platelets per  $\text{mm}^3 \pm \text{s.d.}$  from 5 mice. *b*, Sera were collected at times indicated and assayed at a 10% final concentration on BAF/BO3 and BAF/*mpl* cells. *c*, Sera from 24-h RAMPS-treated mice were adsorbed on either the specific mu-Mpl/Fc or the non-relevant mu4-1BB/Fc fusion proteins. Data are presented as the mean c.p.m. of triplicate wells from which background incorporation was subtracted.

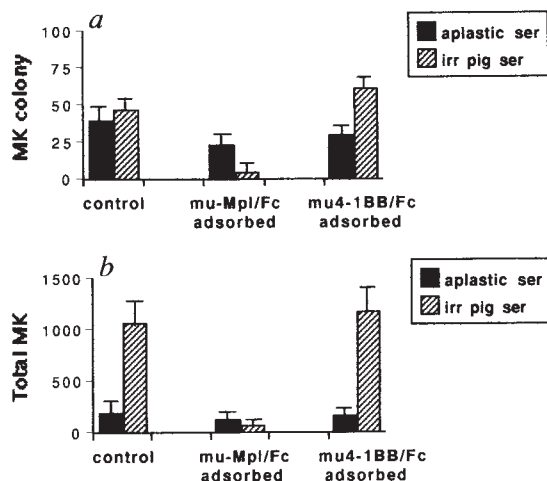


FIG. 4 Effect of serum from a post-irradiated pig on human CFU-MK colony formation. Mononuclear human bone marrow cells were seeded at a concentration of  $2 \times 10^5$  cells per ml in plasma clot cultures in the presence of human aplastic serum or post-irradiated pig serum, untreated or pre-adsorbed on fusion proteins. On day 11 of culture, CFU-MK-derived colonies were visualized by an immunochemical reaction as described<sup>7</sup>. **a**, MK colonies numbers in each culture conditions (mean  $\pm$  s.d. of 3 experiments in duplicate). **b**, Absolute numbers of MK in a total of 12 culture dishes (mean  $\pm$  s.d.).

anti-CD61 monoclonal antibody, stained with propidium iodide and analysed by flow cytometry. The mean absolute number of MK found in cultures with untreated or mu-Mpl/Fc-adsorbed serum was 16,400 and 1,900 per  $1 \times 10^6$  cells, respectively (4 replicate experiments). However, there was no noticeable difference in the modal ploidy distribution because only rare MK (<1%) reached a ploidy level above 16N, as previously reported<sup>14</sup> (data not shown). Thus, on purified CD34-positive cells, Mpl-L in irradiated pig serum appears to act mainly as a potent MK proliferation factor.

In summary, we have demonstrated that the recombinant Mpl-R directly binds a ligand that is present in sera from irradiated animals and transmits a mitogenic signal. However, this observation does not exclude the possibility that other subunits might be needed for full function of Mpl-R as has been demonstrated for other haematopoietin receptors<sup>15,16</sup>. The data provide evidence that Mpl-L is a humoral factor that induces proliferation and differentiation of megakaryocyte progenitors and strongly suggest that Mpl-L plays a pivotal role in regulating platelet homeostasis. The full spectrum of activities of Mpl-L will be precisely defined when the recombinant molecule becomes available and can be used in single cell assays. □

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## The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA

Shou Waga, Gregory J. Hannon\*, David Beach\* & Bruce Stillman

Cold Spring Harbor Laboratory, and \*Howard Hughes Medical Institute, PO Box 100, Bungtown Road, Cold Spring Harbor, New York 11724, USA

THE p53 tumour-suppressor protein controls the expression of a gene encoding the p21 cyclin-dependent protein kinase (CDK) regulator<sup>1–6</sup>. Levels of p21 protein are increased in senescent cells and p21 overexpression blocks the growth of tumour cells<sup>1,3,7</sup>. In normal human cells, but not in many tumour cells, p21 exists in a quaternary complex with a cyclin, a CDK, and the proliferating-cell nuclear antigen (PCNA)<sup>5,8</sup>. p21 controls CDK activity, thereby affecting cell-cycle control<sup>2–4,6</sup>, whereas PCNA functions in both DNA replication<sup>9–12</sup> and repair<sup>13</sup>. Here we use simian virus 40 DNA replication *in vitro* to show that p21 directly inhibits PCNA-dependent DNA replication in the absence of a cyclin/CDK. Furthermore, p21 blocks the ability of PCNA to activate DNA polymerase  $\delta$ , the principal replicative DNA polymerase. This regulation results from a direct interaction between p21 and PCNA. Thus, during p53-mediated suppression of cell proliferation, p21 and PCNA may be important for coordinating cell-cycle progression, DNA replication and repair of damaged DNA.

To understand how p21 suppresses cell proliferation, we investigated whether p21 would affect SV40 DNA replication *in vitro* (reviewed in ref. 14–16). This cell-free system models eukaryotic DNA replication (reviewed in refs 14–17) and enzymatic synthesis of DNA dependent on the SV40 *ori* has been reconstituted using SV40 T-antigen and human DNA replication proteins<sup>12,18</sup>. We expressed histidine-tagged p21 (His-p21; Fig. 1a) and p21 fused to glutathione *S*-transferase (GST) (GST-p21) (data not shown) in *Escherichia coli*. Both purified forms of p21 inhibited CDK (data not shown) and DNA replication from the SV40 *ori* in a crude extract from human 293 cells<sup>19</sup> (Fig. 1b, and data not shown). This latter inhibition was dose-dependent and incomplete, even with large amounts of protein. Heat-treated His-p21, which still functions as a CDK inhibitor<sup>6</sup>, could also inhibit DNA replication, arguing that inhibition was not due to a contaminating nuclease or other enzyme.

Circular DNA topoisomers (the final replication product) and DNA replication intermediates (which migrate more slowly during electrophoresis) disappeared as His-p21 was increased (Fig. 1c). Saturating amounts of His-p21 gave rise to a smear on the gel corresponding to a size of ~2.3 kilobases, which represents accumulation of early DNA-replication intermediates<sup>10,11</sup> (Fig. 1c, lanes 5 and 6). This indicated that p21 could be inhibiting elongation but not initiation of DNA replication. A specific inhibitor, p16, of the cyclin D-dependent kinase CDK4 (ref. 20), did not prevent DNA replication *in vitro*, so this effect must be



specific to p21; moreover, it was also prevented by GST-p21 but not by unrelated GST-fusion proteins (data not shown).

To identify the specific target for p21-mediated inhibition of DNA replication, purified replication proteins were added to the reactions to determine whether they could counteract the inhibition by His-p21. Of these proteins, only PCNA could restore DNA replication in a dose-dependent manner (Fig. 2a).

Neither DNA polymerase  $\delta$  (pol  $\delta$ ) nor replication factor C (reviewed in refs 15, 16) altered the His-p21 effect (Fig. 2a), even though enough of each protein was used to support SV40 DNA replication (see later). When PCNA was added to reactions containing His-p21, circular DNA topoisomers were recovered (Fig. 2b, lanes 3–6), indicating that replication was fully restored. Replication protein A (refs 15, 16) stimulated DNA synthesis

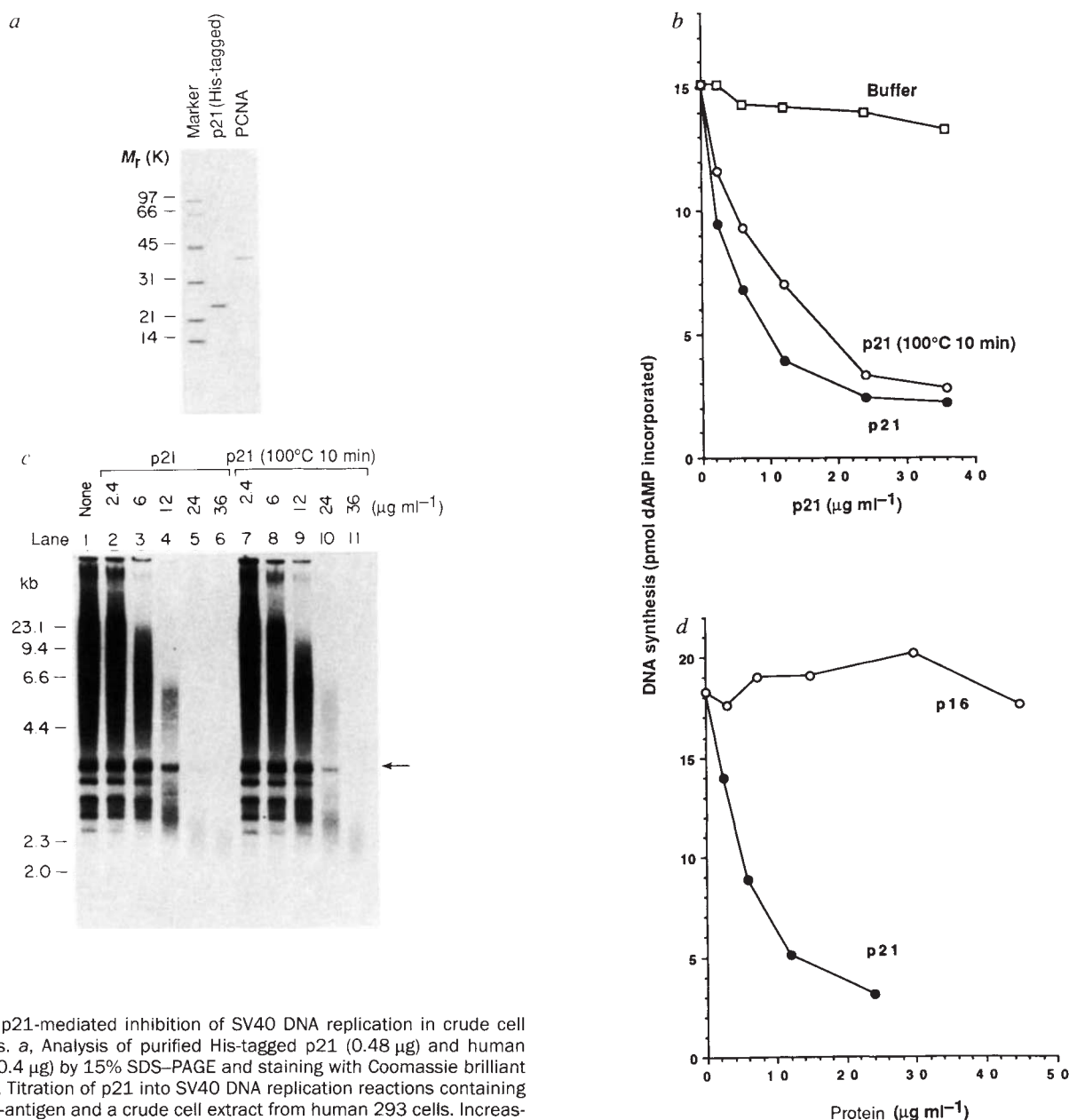


FIG. 1 p21-mediated inhibition of SV40 DNA replication in crude cell extracts. *a*, Analysis of purified His-tagged p21 (0.48  $\mu\text{g}$ ) and human PCNA (0.4  $\mu\text{g}$ ) by 15% SDS-PAGE and staining with Coomassie brilliant blue. *b*, Titration of p21 into SV40 DNA replication reactions containing SV40 T-antigen and a crude cell extract from human 293 cells. Increasing amounts of untreated ( $\bullet$ ) or heat-treated (100  $^{\circ}\text{C}$ , 10 min) ( $\circ$ ) His-p21 were added to replication reactions, using an equivalent amount of buffer as a control ( $\square$ ). The amount of DNA synthesis is expressed as picomoles of dAMP incorporated in a 50- $\mu\text{l}$  reaction in 1 hour. *c*, Analysis by electrophoresis on 1% agarose gels and autoradiography of products purified from each reaction in *b*. Lane 1, buffer control of same volume as p21 in lanes 6 and 11. Arrow indicates form-I relaxed DNA; other bands are form-I topoisomers. *d*, Comparative effects on DNA replication of His-p21 and His-p16 CDK inhibitors: increasing protein was added to replication reactions reconstituted with crude cell extract from human 293 cells.

**METHODS.** DNA was replicated from the SV40 origin in 10  $\mu\text{l}$  containing 6  $\mu\text{g ml}^{-1}$  pSV011 plasmid DNA<sup>10</sup>, 44  $\mu\text{g ml}^{-1}$  SV40 T-antigen, 1.5  $\text{mg ml}^{-1}$  protein of a crude S100 cell extract from 293 cells as described<sup>19</sup>. Crude extracts were preincubated on ice for 30 min with

His-p21 or His-p16 without SV40 T-antigen or plasmid DNA, then incubated at 37  $^{\circ}\text{C}$  for 1 h with T-antigen and DNA template. Measurement of incorporated radioactivity and the analysis of replication products have been described<sup>18,29</sup>. His-p21 contained an insertion (Gly-His<sub>6</sub>) between amino acids 1 and 2 of the p21 sequence. It was expressed in bacteria and purified on fast-flow S-Sepharose (0–1 M NaCl gradient in 20 mM Tris, pH 8.0). p21-containing fractions were diluted 3-fold with 20 mM Tris, pH 8.0, 10% glycerol, and passed through fast-flow Q-Sepharose. Flow-through material was loaded onto Ni-NTA agarose (Qiagen) and His-p21 was eluted with 100 mM imidazole in 20 mM Tris, pH 8.0. Baculovirus-expressed, His-tagged p16 was provided by M. Serrano and D.B. (ref. 20). Human PCNA was expressed in *E. coli* and purified as before<sup>18</sup>; the other DNA replication proteins have been described<sup>12,18,20,29</sup>.

in the presence of His-p21, but only early intermediates were formed (S.W. and B.S., unpublished results). These results indicated that p21 was interfering with PCNA function during DNA replication. The human cell crude extract contained cyclin-CDK activity and therefore, it was possible that the p21 inhibition of DNA replication was indirect and involved its CDK inhibitory activity.

This possibility was investigated by examining the effect of p21 on SV40 *ori*-dependent DNA replication reconstituted with purified replication proteins, which did not include cyclins and CDKs, instead of the crude cell extract. Coordinated replication of both lagging and leading DNA strands occurs in this system<sup>11,12</sup>. We found that His-p21 again inhibited DNA replication (Fig. 3a) and that this inhibition could be reversed by the

addition of excess PCNA (Fig. 3b). These results indicate that p21 and PCNA interact with one another directly, which was confirmed by analysis of DNA polymerase  $\delta$ -directed DNA synthesis using an oligo(dT)/poly(dA) primer-template DNA. DNA polymerase  $\delta$  needs PCNA for processive polymerase activity<sup>9,21</sup>, and both are required for replication of the lagging and leading DNA strands<sup>10,12</sup>. Purified PCNA stimulated DNA synthesis by purified pol  $\delta$  more than 10-fold in the absence of p21, but this was dramatically suppressed by His-p21 (Fig. 3c) and by GST-p21 (data not shown). Even with saturating amounts of His-p21, however, DNA synthesis by pol  $\delta$  did not fall below the level achieved without PCNA, suggesting that p21 does not affect pol  $\delta$  itself. An excess of PCNA, but not of pol  $\delta$ , reversed this inhibition (Fig. 3d).

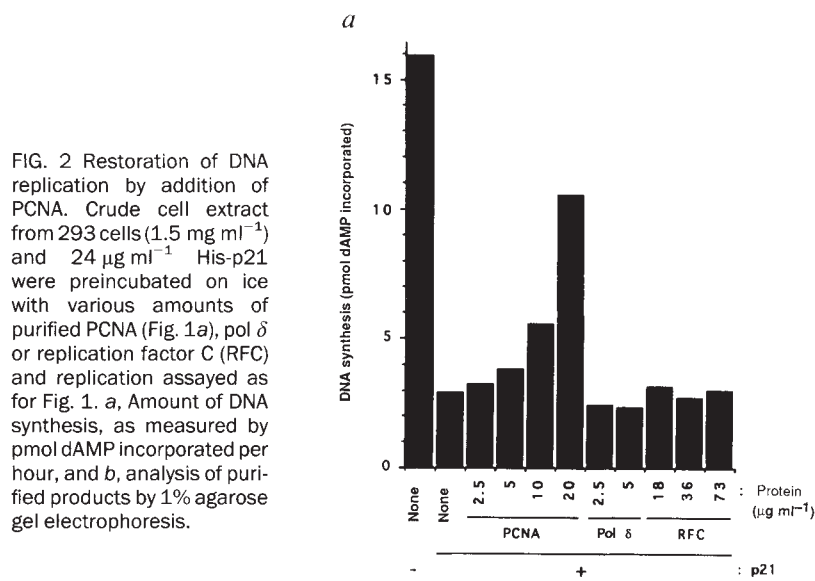
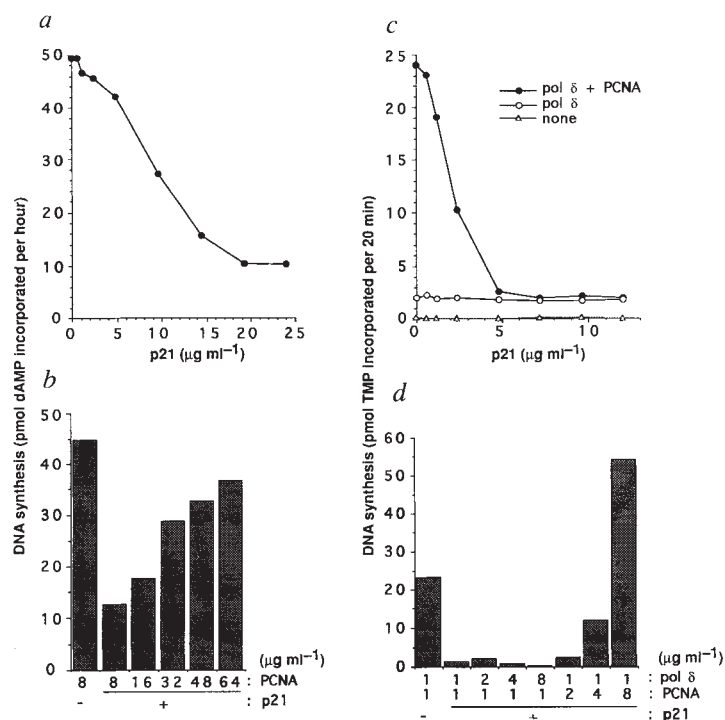
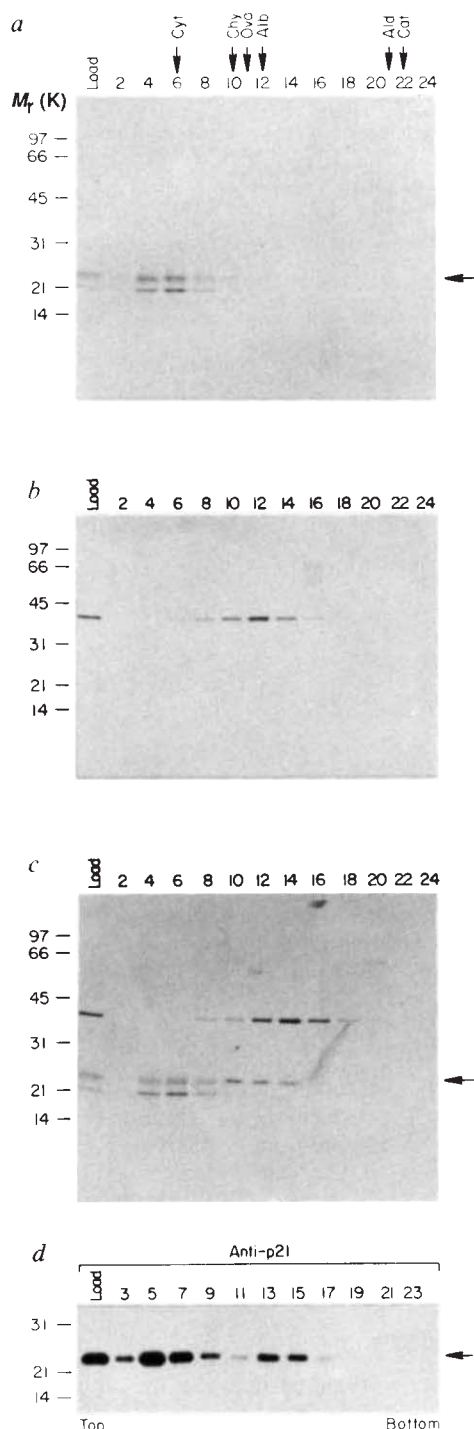


FIG. 3 Inhibition by His-p21 of DNA synthesis using purified replication proteins. a and b, Analysis of the reconstituted SV40 DNA replication system. Purified proteins were used for reconstituted replication including replication protein (RPA), topoisomerases I and II, RFC (fraction IV), DNA polymerase- $\alpha$ /primase complex (pol  $\alpha$ /primase), PCNA and pol  $\delta$ . These proteins were preincubated on ice with His-p21 (a) or with PCNA and  $19 \text{ } \mu\text{g ml}^{-1}$  His-p21 (b), T-antigen and plasmid DNA were then added and replication assayed as for Fig. 1. c and d, Analysis of PCNA-activated DNA synthesis by purified DNA polymerase  $\delta$  with oligo(dT)/poly(dA) as primer-template. DNA synthesis is expressed as picomoles TMP incorporated in a 25- $\mu\text{l}$  reaction in 20 min. c, His-p21 was preincubated with  $1 \text{ } \mu\text{g ml}^{-1}$  PCNA and  $1 \text{ } \mu\text{g ml}^{-1}$  pol  $\delta$  (filled circles), pol  $\delta$  alone (circles) or control buffer (triangles) on ice for 30 min, then incubated with TTP and the homopolymer primer-template DNA at  $37^\circ\text{C}$  for 20 min. d, PCNA and pol  $\delta$  were incubated with or without  $6 \text{ } \mu\text{g ml}^{-1}$  His-p21 on ice before DNA synthesis.

METHODS. Reconstituted replication and DNA synthesis reactions with pol  $\delta$  were carried out as described<sup>18,21,29</sup>. The standard reconstituted replication reaction contained  $6 \text{ } \mu\text{g ml}^{-1}$  pSV011 template plasmid,  $80 \text{ } \mu\text{g ml}^{-1}$  T-antigen,  $4 \text{ } \mu\text{g ml}^{-1}$  topoisomerase I,  $1.8 \text{ } \mu\text{g ml}^{-1}$  topoisomerase II,  $50 \text{ } \mu\text{g ml}^{-1}$  RPA,  $8 \text{ } \mu\text{g ml}^{-1}$  PCNA,  $72 \text{ } \mu\text{g ml}^{-1}$  RFC (fraction IV),  $1 \text{ } \mu\text{g ml}^{-1}$  pol  $\alpha$ /primase complex and  $5 \text{ } \mu\text{g ml}^{-1}$  pol  $\delta$ . These proteins were purified as before<sup>18,29</sup>.







These results encouraged us to determine whether p21 and PCNA could form a complex in solution in the absence of CDKs or cyclins using glycerol gradient sedimentation. His-p21 sedimented at the same position as a marker protein of relative molecular mass 12,500 ( $M_r$ , 12.5K) (fraction 6 in Fig. 4a); PCNA co-sedimented with a 68K marker protein (fraction 12 in Fig. 4b) (PCNA forms a torus-like homo-trimer in solution<sup>22,23</sup>). When PCNA and His-p21 were mixed before applying to the gradient, the PCNA peak shifted to fraction 14 and cosedimented with part of the His-p21 (Fig. 4c). Note that a contaminating *E. coli* protein present in this His-p21 preparation did not bind PCNA. Immunoblotting with p21 antiserum confirmed that the PCNA-associated protein was His-p21 (Fig. 4d). The increased sedimentation constant of PCNA when bound to His-p21 or GST-

FIG. 4 Analysis of a His-p21/PCNA complex by glycerol gradient sedimentation. His-p21 (a), PCNA (b) or both proteins combined (c, d) were sedimented in parallel in 15–35% glycerol gradients containing 0.25 M NaCl. Proteins in every second fraction (out of 27 per gradient) were precipitated with trichloroacetic acid, separated by 15% SDS-PAGE, and stained with silver (top three panels). Alternative fractions containing both His-p21 and PCNA were analysed by western blotting with rabbit anti-p21 antiserum (G.H. and D.B., unpublished) (panel d). The fraction numbers are shown above each gel. Protein in the 'Load' lanes is equivalent to one fifteenth of the total protein in each gradient. Sedimentation positions of the marker proteins, cytochrome c (Cyt; 12.5K), chymotrypsinogen A (Chy; 25K), ovalbumin (Ova; 45K), serum albumin (Alb; 68K), aldolase (Ald; 158K) and catalase (Cat; 240K) are indicated in a. Migration positions of size markers in SDS-PAGE are shown on the left. Arrows in a, c and d indicate His-p21. The protein migrating faster than His-p21 is an unrelated *E. coli* protein that serves as an internal control; bands at ~66K in b and c are contaminants introduced in the gel electrophoresis.

**METHODS.** A mixture of PCNA and His-p21 (0.8  $\mu$ g each, giving a molar excess of p21) or each protein alone was preincubated on ice for 2 h in a buffer consisting of 0.25 M NaCl, 25 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40 and protease inhibitors, before sedimentation in 5-ml 15–35% glycerol gradients in the same buffer in a Beckman SW55Ti rotor for 21.5 h at 55,000 r.p.m. at 4 °C. A duplicate gradient was run in parallel with protein markers. Immunoblotting with rabbit anti-p21 antiserum followed the manufacturer's recommended protocol (ECL, Amersham).

p21 (data not shown) suggests that one molecule of p21 is bound to one molecule of a trimeric PCNA.

In normal non-tumorigenic human cells, p21 exists in a quaternary complex with a cyclin, a CDK and PCNA<sup>8,24</sup>. Because p21 binds to PCNA directly, a complex of p21 with only PCNA may exist in normal cells. The activity of the kinase in the quaternary complex depends on the amount of p21 in the complex (H. Zhang, G.J.H. and D.B., manuscript submitted), so in normal cells p21 probably controls both CDK/cyclin and PCNA activity. Thus p21 could provide a regulatory link in normal diploid cells between DNA replication and those CDK/cyclin complexes required for the execution of S phase. It has been proposed that PCNA in *S. pombe* participates in a replication checkpoint that affects entry into mitosis<sup>25</sup>, so an alternative role for the p21/PCNA complex or the quaternary complex at the replication fork might be in mediating this checkpoint.

The level of p21 is controlled by the tumour-suppressor protein p53 (refs 1, 4), and our observations suggest a mechanism whereby p53 could modulate and perhaps coordinate cell-cycle progression and DNA replication. In tumour cells that have either lost the p53 protein or contain an altered form of p53, p21 levels are greatly reduced or the protein is absent altogether<sup>1,4,5</sup>. The consequences of this could be abnormal DNA replication control or loss of coordination between DNA replication and cell-cycle progression. Both could lead to genome instability, a characteristic of tumour cells.

Following DNA damage by ultraviolet light,  $\gamma$ -rays or chemicals, cells respond by activating DNA repair processes that are partly controlled by p53<sup>26–28</sup>. Induction of p21 by p53 could be one mechanism by which the cell cycle<sup>1,3,4,6</sup> and DNA replication are transiently arrested. As PCNA is required for DNA-excision repair<sup>13</sup>, we assume either that the PCNA and DNA polymerase involved in DNA repair are refractory to inhibition by p21, or that there is a damage-induced programme that allows DNA repair before resumption of replication.

In addition to its established role as a CDK inhibitor, our results demonstrate a previously unrecognized function for p21 in the control of DNA replication. This suggests that p21 may be a key governor of DNA replication, DNA repair and the cell cycle machinery. □

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## RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription

Sami N. Guzder, Patrick Sung, Véronique Bailly, Louise Prakash & Satya Prakash\*

Sealy Center for Molecular Science,  
University of Texas Medical Branch,  
6.104 Medical Research Building, Galveston,  
11th and Mechanic Street, Texas 77555-1061, USA

THE *RAD25* gene of *Saccharomyces cerevisiae* functions in nucleotide excision repair of ultraviolet-damaged DNA and is also required for cell viability<sup>1</sup>. The RAD25 protein shows remarkable homology to the protein encoded by the human nucleotide-excision-repair gene *XPB* (*ERCC3*), mutations in which cause the cancer-prone disease xeroderma pigmentosum and also Cockayne's syndrome<sup>1</sup>. Here we purify RAD25 protein from *S. cerevisiae* and show that it contains single-stranded DNA-dependent ATPase and DNA helicase activities. Extract from the conditional lethal mutant *rad25-ts<sub>24</sub>* exhibits a thermolabile transcriptional defect which can be corrected by the addition of RAD25 protein, indicating a direct and essential role of RAD25 in RNA polymerase II transcription. The protein encoded by the *rad25<sup>799am</sup>* allele is defective in DNA repair but is proficient in RNA polymerase II transcription, indicating that RAD25 DNA-repair activity is separable from its transcription function. The *rad25 Arg-392* encoded product, which contains a mutation in the ATP-binding motif, is defective in RNA polymerase II transcription, suggesting that the RAD25-encoded DNA helicase functions in DNA duplex opening during transcription initiation.

The RAD25 (SSL2) protein was overproduced in *S. cerevisiae* (Fig. 1a), and purified almost to homogeneity (Fig. 1b). RAD25 protein in the overproducing extract (Fig. 1a, lane 2) and RAD25 protein immunoprecipitated from wild-type extract (Fig. 3a, lane 1) both show an apparent molecular size of 105K in SDS-PAGE analysis, which is consistent with the predicted size of 95.3K<sup>1</sup>. The identity of purified RAD25 protein was established by immunoblotting (Fig. 1a, lane 3).

To examine whether RAD25 protein has ATPase activity, it was incubated with [2,5,8-<sup>3</sup>H]ATP in the absence of DNA and with either double-stranded (ds) or single-stranded (ss) DNA as cofactor. As shown in Table 1, RAD25 hydrolyses ATP to ADP in the presence of DNA but not in its absence, with ssDNA being much more effective than dsDNA in activating ATPase activity. The ssDNA-dependent ATPase activity of RAD25 pro-

TABLE 1 RAD25 protein is a ssDNA-dependent ATPase

| Conditions                 | ATPase activity (%) |
|----------------------------|---------------------|
| No DNA                     | 0                   |
| Single-stranded DNA        |                     |
| M13 mp18                   | 100                 |
| ΦX174                      | 100                 |
| ΦX174 (–Mg <sup>2+</sup> ) | 0                   |
| Duplex DNA                 |                     |
| pBR322                     | 5                   |
| M13mp18                    | 7                   |
| ΦX174                      | 8                   |
| ΦX174 (–Mg <sup>2+</sup> ) | 0                   |

Reaction mixtures (10 µl final volume) containing 20 ng RAD25 protein, 200 ng of the indicated DNA co-factor, and 0.25 mM [2,5,8-<sup>3</sup>H]ATP (10<sup>5</sup> c.p.m. total) were assembled in 30 mM Tris-HCl, pH 7.25, containing 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol and 100 µg ml<sup>–1</sup> BSA and incubated for 30 min at 36 °C. The duplex DNA contained 80% supercoiled form and 20% nicked circular form. Conversion of ATP to ADP was determined by thin layer chromatography in polyethyleneimine cellulose sheets; 100% activity corresponds to the hydrolysis of 35% of the input ATP.

tein requires Mg<sup>2+</sup> (Table 1), is maximal at pH 7.25 (data not shown), and has *k<sub>cat</sub>* = 140 min<sup>–1</sup> at this pH. The level of ssDNA-dependent ATPase activity in the Mono-Q fractions from the last step of RAD25 purification was found to parallel closely the amount of RAD25 (Fig. 2a), indicating that this activity is intrinsic to RAD25 protein. RAD25 protein also hydrolyses dATP with similar efficiency and the same DNA cofactor requirement (data not shown).

Many ssDNA-dependent ATPases have the ability to unwind duplex DNA<sup>2</sup>. To examine whether RAD25 has DNA helicase activity, we incubated it with ssM13 circular DNA to which a <sup>32</sup>P-labelled 17-nucleotide complementary fragment had been annealed. Figure 2b shows that RAD25 protein unwinds the partial duplex (lanes 4 and 5) in an ATP-dependent manner (compare lane 3 with lane 5). Replacing ATP with the non-hydrolysable ATP analogue ATP-γS inactivates DNA unwinding (lane 6), suggesting that the energy derived from hydrolysing the β-γ phosphodiester bond in ATP drives the helicase function. Whereas dATP (lane 7) substitutes for ATP in supporting DNA unwinding, CTP (lane 8), GTP (lane 9), UTP (lane 10) and ADP (data not shown) are ineffective. As shown in Fig. 2c, RAD25 protein also catalyses the ATP-dependent unwinding of another helicase substrate which contains a 41-base-pair (bp) duplex region. The helicase activity requires Mg<sup>2+</sup>, has a pH 7.25 optimum, and co-elutes with RAD25 protein during chromatography in Mono-Q (data not shown).

Our previous studies with the recessive temperature-sensitive (*ts*) conditional lethal mutation *rad25-ts<sub>24</sub>* have indicated a general requirement for RAD25 in RNA polymerase II transcription *in vivo*<sup>3</sup>. Here we determine whether RAD25 functions directly in RNA polymerase II transcription. Using immunopre-

\* To whom correspondence should be addressed.



cipitation, we find that the *rad25-ts<sub>24</sub>*-encoded protein is the same size as the wild-type RAD25 (Fig. 3a; compare lanes 1 and 2). To investigate whether the *in vivo* transcriptional defect of *rad25-ts<sub>24</sub>* could be due to a direct involvement of the wild-type RAD25 protein in RNA polymerase II transcription, we examined the transcription of the template pSL187 containing the yeast *CYC1* TATA element and a 400-bp G-less region<sup>4</sup> in extracts<sup>5</sup> prepared from *rad25-ts<sub>24</sub>* cells and from isogenic wild-type RAD25 cells

grown at 25 °C. Figure 3b shows that extract from *rad25-ts<sub>24</sub>* cells carries out transcription at a reduced rate compared with extract from the wild-type strain. Whereas pretreatment of wild-type extract at 37 °C for 5 min does not affect its transcriptional competence (Fig. 3c), the same heat treatment completely abol-

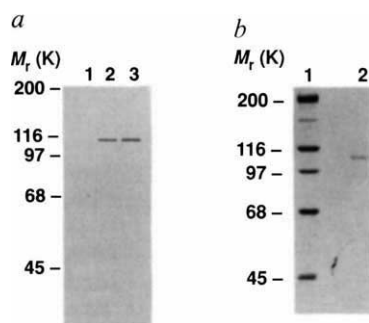


FIG. 1 Overproduction and purification of RAD25 protein. *a*, The nitrocellulose blot of a 7% denaturing polyacrylamide gel was probed with affinity-purified anti-RAD25 antibodies. Lane 1, extract from yeast strain LP2749-9B containing the vector pMA91 which has the *PGK* promoter but lacks the *RAD25* gene. Lane 2, extract from yeast strain LP2749-9B containing the *RAD25*-overproducing plasmid pPM318. Lane 3, 3 ng purified RAD25 protein. *b*, A denaturing polyacrylamide gel containing 500 ng purified RAD25 protein (lane 2) and size markers (lane 1) was stained with Coomassie blue R.

**METHODS.** A hybrid polypeptide consisting of amino-acid residues 253–790 of RAD25 protein fused to the N-terminal 48 amino acids of the *Escherichia coli* transcription termination factor  $\rho$  was expressed in *E. coli*, purified from inclusion bodies by preparative SDS-PAGE, and used for raising polyclonal antiserum in rabbits. Antibodies were affinity-purified from serum using a Sepharose column containing covalently coupled  $\rho$ -RAD25 hybrid protein. For expressing RAD25 protein in *S. cerevisiae*, the entire open reading frame of RAD25 was placed downstream of the *PGK* promoter in the vector pMA91 (2 $\mu$ , *PGK*) to yield the plasmid pPM318 (2 $\mu$ , *PGK*-RAD25). Extract was prepared from 1 kg of the protease-deficient yeast strain LP2749-9B harbouring pPM318 as described<sup>15</sup>. The supernatant obtained from ultracentrifugation (100,000g, 90 min) was treated with 0.22 g ml<sup>-1</sup> ammonium sulphate to precipitate RAD25 protein. The protein pellet was dissolved in 500 ml buffer K (20 mM potassium phosphate, pH 7.5, containing 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol) and dialysed, with one change, against a total of 10 l buffer K containing 50 mM KCl. The dialysate (fraction I; 600 ml) was applied onto a Q-Sepharose column (2.5  $\times$  24 cm), which was eluted with a 1.2-l gradient of 60–550 mM KCl. The fractions containing RAD25 protein, eluting at ~330 mM KCl as identified by immunoblotting, were pooled (fraction II; 90 ml) and treated with 0.24 g ml<sup>-1</sup> ammonium sulphate. The precipitated proteins were pelleted by centrifugation (20,000g, 30 min), dissolved in 60 ml buffer K and dialysed against 2 l of buffer K for 4 h. The dialysate (ionic strength equivalent to 40 mM KCl) was chromatographed in an S-Sepharose column (1.5  $\times$  8 cm) with a 200-ml gradient of 0–300 mM KCl. RAD25 protein eluted from S-Sepharose at 110 mM KCl was pooled (fraction III; 12 ml) and dialysed against buffer K to lower the ionic strength to 40 mM before being fractionated in Mono S (HR5/5) using a 20-ml 50–300 mM KCl gradient. The RAD25 pool (fraction IV; 2 ml, ionic strength 120 mM KCl) was concentrated to 1 ml and sized on a Sephacryl S-300 column (1  $\times$  45 cm) in buffer K plus 100 mM KCl. The RAD25-containing fractions were pooled (fraction V; 3.5 ml) and further chromatographed in Mono-Q (HR5/5) using a 24-ml KCl gradient from 100–500 mM, collecting 1-ml fractions. The RAD25 peak and surrounding fractions were concentrated individually in Centricon-30 to 100  $\mu$ l. RAD25 protein (fraction VI) from the Mono-Q step was nearly homogeneous as determined by Coomassie-blue staining after SDS-PAGE (*b*). The yield of highly purified RAD25 protein from the starting 1 kg of yeast paste was 6  $\mu$ g.

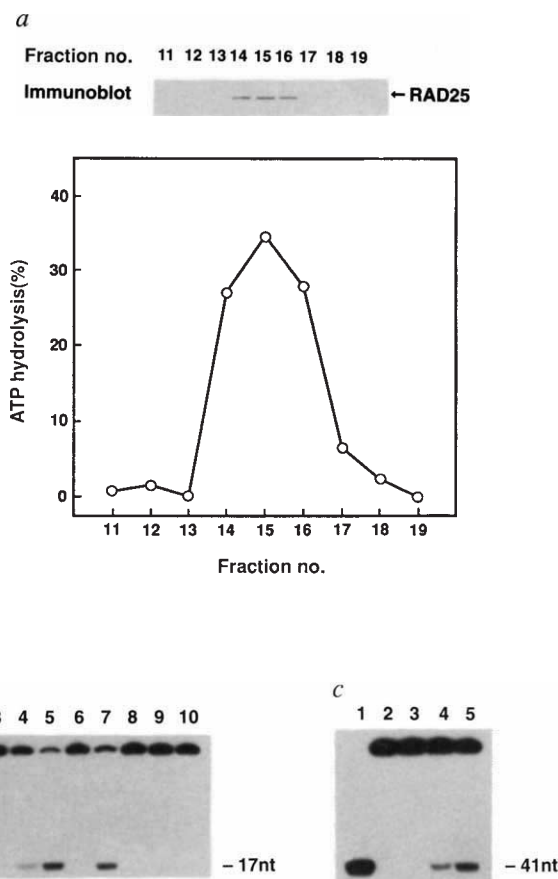


FIG. 2 ATP hydrolysis and DNA unwinding by RAD25 protein. *a*, Concentrated Mono-Q fractions 11–19 from RAD25 protein purification were examined for their RAD25 content by immunoblotting and for ssDNA-dependent ATP hydrolysis as described in Table 1 legend. *b*, RAD25 protein is a DNA helicase. The 17-bp partial duplex was incubated in ATP-containing buffer without RAD25 protein (lane 2) or with 10 ng (lane 4) and 20 ng (lane 5) of RAD25 protein. The DNA substrate was also incubated with 20 ng RAD25 in the absence of any nucleotide (lane 3) and in the presence of ATP- $\gamma$ S (lane 6), dATP (lane 7), CTP (lane 8), GTP (lane 9) or UTP (lane 10). Lane 1 contains heat-denatured substrate. *c*, ATP-dependent unwinding of a 41-bp partial duplex. The DNA substrate (lane 2) was incubated with 10 ng (lane 4) or 20 ng (lanes 3 and 5) RAD25 protein in the absence (lane 3) or presence of ATP (lanes 4 and 5). Lane 1 contains heat-denatured substrate.

**METHODS.** In *a*, the indicated Mono-Q fractions from RAD25 purification were concentrated individually from 1 ml to 100  $\mu$ l using Centricon-30 (Fig. 1 legend), and 0.2  $\mu$ l and 1  $\mu$ l of the concentrated fractions were used for immunoblotting and for ATPase assay, respectively. The helicase substrates were constructed by hybridizing a <sup>32</sup>P-labelled 17-nucleotide fragment complementary to positions 6,310–6,326 or a <sup>32</sup>P-labelled 41-base fragment complementary to positions 6,822 to 6,862 of the viral (+) strand of M13 and purifying the resulting partial duplexes as described<sup>16</sup>. Helicase reaction mixtures (10  $\mu$ l) were assembled in 30 mM Tris-HCl, pH 7.25, containing 5 mM MgCl<sub>2</sub>, 60  $\mu$ g ml<sup>-1</sup> BSA, 1 mM DTT, 30 mM KCl, 1.5 mM ATP or one of the other nucleotides as indicated, and RAD25 protein. After 30 min at 36 °C, the reaction was terminated by addition of 5  $\mu$ l of 1% SDS, 50 mM EDTA, 20% glycerol, and 0.02% bromophenol blue. Displacement of the hybridized <sup>32</sup>P-labelled fragment from the helicase substrates was detected by autoradiography after electrophoresis of the reaction mixtures in polyacrylamide gels<sup>16</sup>.

ishes the ability of *rad25-ts<sub>24</sub>* extract to synthesize messenger RNA (Fig. 3c), indicating that RNA polymerase II transcription is rendered thermolabile by the *rad25-ts<sub>24</sub>* mutation. To test whether the *in vitro* transcriptional defect in the *rad25-ts<sub>24</sub>* mutant extract can be corrected by addition of RAD25, increasing amounts of purified RAD25 were added to transcription reaction mixtures containing wild-type and *rad25-ts<sub>24</sub>* extracts which had or had not been pretreated at 37 °C for 5 min. The addition of 2.5–7.5 ng of RAD25 protein to untreated (Fig. 3d) or heat-treated (Fig. 3e) wild-type extract does not affect transcription. Addition of RAD25 to the untreated (Fig. 3d) or heat-treated (Fig. 3e) *rad25-ts<sub>24</sub>* extract restores transcription, so that wild-type levels of transcriptional activity are seen in the *rad25-ts<sub>24</sub>* extracts at 7.5 ng RAD25 (Fig. 3d and e). These observations indicate a direct role of RAD25 in RNA polymerase II transcription.

During transcription by RNA polymerase II, conversion of the preinitiation complex to the open complex capable of initiating mRNA synthesis requires the hydrolysis of the  $\beta$ - $\gamma$  bond of ATP<sup>6,7</sup>. Here we test the possibility that the ATPase/DNA helicase activity of RAD25 is required for RNA polymerase II transcription. We found previously that mutating the lysine 392 residue to arginine (*rad25 Arg-392*) in the Walker type-A nucleotide-binding motif of RAD25 is lethal, suggesting an essential role for RAD25 ATPase/helicase activity<sup>8</sup>. In RAD3, a similar mutation of the lysine residue to arginine (*rad3 Arg-48*) in the Walker type-A motif inactivates the ATPase and DNA helicase activities<sup>9</sup>. To determine whether the *rad25 Arg-392* encoded protein is defective in RNA polymerase II transcription *in vitro*, we first verified that the *rad25 Arg-392* mutant gene expresses a

protein by introducing the low-copy centromeric plasmid pPM147 carrying the *rad25 Arg-392* gene into the strain EPY88, which harbours the *rad25<sup>799am</sup>* mutant gene in the genome. The *rad25<sup>799am</sup>* mutation, which deletes 45 amino acids of RAD25 protein at the carboxy terminus, renders cells ultraviolet-sensitive but has no effect on cell viability<sup>8</sup>. As expected, the *rad25<sup>799am</sup>*-encoded protein is smaller than the wild-type RAD25 protein (Fig. 3a; compare lanes 1 and 3). In cells carrying both mutant alleles, *rad25<sup>799am</sup>* and *rad25 Arg-392*, the proteins encoded by these two alleles can be easily distinguished because of their size difference. As shown in Fig. 3a, the *rad25 Arg-392* protein is expressed and is the same size as wild-type RAD25 (upper band in lane 4). For RNA polymerase II transcription, extracts from wild-type *RAD25* cells, from *rad25-ts<sub>24</sub>* cells with or without pPM147, which expresses the Rad25 Arg-392 mutant protein, and from the *rad25<sup>799am</sup>* mutant with or without plasmid pPM147, were examined for the transcription of the template pSL187 directly (Fig. 3f, lanes 1 to 5), or the various extracts were subjected to a 5-min treatment at 37 °C before their use in the transcription reaction (Fig. 3f, lanes 6 to 10). The presence of the Rad25 Arg-392 mutant protein does not enhance the transcriptional activity of the *rad25-ts<sub>24</sub>* extract, regardless of whether the extract has been heat-treated or not (Fig. 3f; compare lanes 5 and 10 with lanes 4 and 9). As the *RAD25* gene carried on a low-copy centromeric plasmid restores normal transcription in the *rad25-ts<sub>24</sub>* extract (data not shown), the failure of the Rad25 Arg-392 mutant protein to correct the transcriptional defect of the *rad25-ts<sub>24</sub>* extract (Fig. 3f) indicates that the Rad25 Arg-392 protein is defective in transcription. Interestingly, the *rad25<sup>799am</sup>* extract is fully competent for RNA

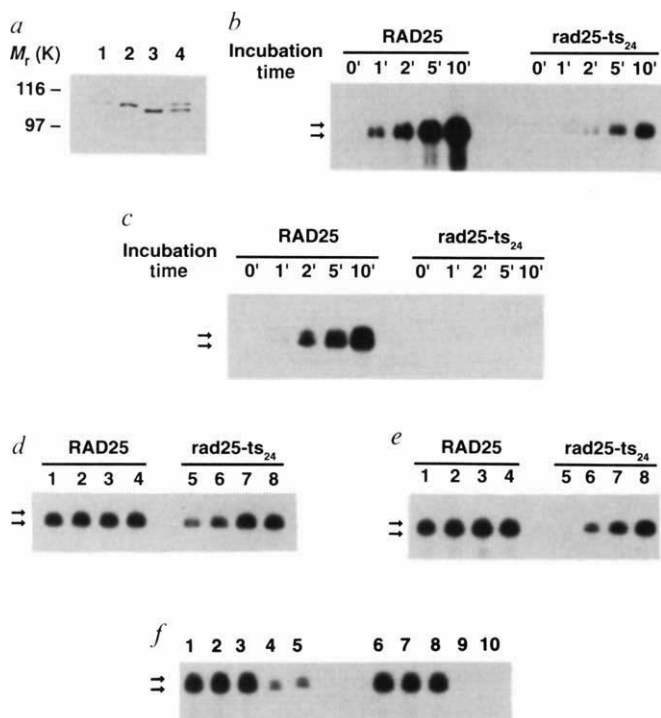


FIG. 3 RAD25 helicase is required for RNA polymerase II transcription. **a**, Immunoprecipitation of various Rad25 mutant proteins. Wild-type RAD25 protein (lane 1), Rad25-*ts<sub>24</sub>* protein (lane 2), Rad25<sup>799am</sup> protein (lane 3), Rad25 Arg-392 protein (upper band in lane 4), together with Rad25<sup>799am</sup> protein (lower band in lane 4) were isolated by immunoprecipitation from the appropriate yeast extracts and examined by immunoblotting. **b** and **c**, RNA polymerase II transcription in *rad25-ts<sub>24</sub>*

extract is thermolabile. Extracts from isogenic wild-type and *rad25-ts<sub>24</sub>* cells were examined for RNA polymerase II transcriptional activity without (**b**) or with (**c**) a 5-min pretreatment of the extracts at 37 °C. Transcription reactions were carried out at 22 °C for the times indicated. **d** and **e**, Purified RAD25 protein corrects the transcriptional defect in *rad25-ts<sub>24</sub>* extract. Wild-type and *rad25-ts<sub>24</sub>* extracts without (**d**) or with (**e**) a 5-min pretreatment at 37 °C were examined for RNA polymerase II transcription in the absence (lanes 1 and 5) or presence of 2.5 ng (lanes 2 and 6), 5 ng (lanes 3 and 7) and 7.5 ng (lanes 4 and 8) of purified RAD25 protein. Transcription reactions were carried out at 22 °C for 10 min. **f**, The *rad25 Arg-392* mutation inactivates RNA polymerase II transcription. Extracts from wild-type cells (lanes 1 and 6), *rad25<sup>799am</sup>* cells without (lanes 2 and 7) and with plasmid pPM147 that expresses the Rad25 Arg-392 protein (lanes 3 and 8), and from *rad25-ts<sub>24</sub>* cells without (lanes 4 and 9) or with plasmid pPM147 (lanes 5 and 10) were examined for RNA polymerase II transcription directly (lanes 1 to 5) or after a 5-min pretreatment of extracts at 37 °C (lanes 6 to 10). Transcription reactions were carried out at 22 °C for 10 min. Arrows indicate the 375- and 350-nucleotide transcripts<sup>4</sup>.

**METHODS.** For immunoprecipitation, cells were grown at 25 °C. Extracts were prepared from *RAD25*, *rad25-ts<sub>24</sub>* cells and from *rad25<sup>799am</sup>* cells with or without the low-copy plasmid pPM147 containing the *rad25 Arg-392* gene and treated with protein A-agarose beads bearing covalently conjugated anti-RAD25 antibodies as described<sup>26</sup>. The RAD25 and various mutant Rad25 proteins were eluted from immunoprecipitates using 1% SDS<sup>26</sup> and analysed by immunoblotting. Extracts for RNA polymerase II transcription were prepared according to ref. 5. Transcription of the DNA template pSL187 containing the *CYC1* TATA element placed upstream of a 400-bp G-less cassette was examined as described<sup>4,13</sup>. Transcription initiates within the G-less region and results in the synthesis of two <sup>32</sup>P-labelled transcripts 350 nt and 375 nt in length<sup>4</sup>. After incubation for the indicated times at 22 °C, the reaction mixture was treated with 6 units RNase T1, deproteinized, and the RNA transcripts were precipitated overnight at -70 °C with ethanol and 10 µg yeast tRNA as carrier<sup>5</sup>. The transcripts were dissolved in 30 µl loading buffer (80% formamide, 0.1 × TBE, 0.01% xylene cyanol FF, 0.01% bromophenol blue) and 10 µl of this solution was electrophoresed in a 4% polyacrylamide-7 M urea gel. The <sup>32</sup>P-labelled transcripts were visualized by autoradiography of the dried gel.



polymerase II transcription (Fig. 3f, lanes 2 and 7), which is congruent with our previous genetic results, indicating that the *rad25*<sup>799am</sup> mutation affects the ability to repair ultraviolet-damaged DNA but has no effect on cell viability<sup>8</sup>.

Transcription factor b from yeast, factor  $\delta$  from rat and factor TFIIF from humans are functionally equivalent and all contain a DNA-dependent ATPase activity<sup>10–12</sup>. RAD3 has a direct and essential role in RNA polymerase II transcription<sup>13</sup>, and it is part of factor b<sup>14</sup>. RAD25 interacts with factor b<sup>14</sup>. The *XPB* (*ERCC3*)-encoded protein is a constituent of TFIIF<sup>12</sup>, and it is likely that XPD (*ERCC2*), the human counterpart of yeast RAD3, is also a component of TFIIF. A DNA helicase activity has been reported to copurify with TFIIF<sup>12</sup>. We have previously shown that RAD3 and XPD possess ssDNA-dependent ATPase and DNA helicase activities<sup>15–17</sup>. Our finding that RAD25 has ssDNA-dependent ATPase and DNA helicase activities predicts that XPB should also contain similar activities. The ATPase/DNA helicase activities of TFIIF and its counterparts in other eukaryotic organisms are thus expected to derive from a combination of XPB (RAD25) and XPD (RAD3) proteins.

Because the *Rad3 Arg-48* mutant defective in DNA helicase activity retains normal viability, RAD3 DNA helicase activity is dispensable for transcription<sup>9</sup>. In contrast, our studies with the *rad25 Arg-392* mutation<sup>8</sup>, carrying a similar mutation in the RAD25 nucleotide-binding motif to *rad3 Arg-48*, implicate RAD25 DNA helicase activity in its essential function in RNA polymerase II transcription. The DNA helicase activity of RAD25 and its human counterpart XPB may effect duplex melting in the conversion of the preinitiation complex to the open complex during transcription by RNA polymerase II.

Genetic studies have indicated a requirement for both the RAD3 and RAD25 DNA helicase activities in nucleotide excision repair (NER)<sup>8,9</sup>. RAD3 protein functions in DNA damage recognition<sup>18</sup> and its helicase activity is required subsequent to incision of damaged DNA<sup>9</sup>. One possible role of RAD25 DNA helicase activity in NER could be in unwinding the damaged duplex DNA to prepare it for dual incision in the damaged strand by the single-strand-specific RAD1/RAD10 (refs 19, 20) and RAD2 endonucleases<sup>21</sup>.

DNA lesions are removed at a faster rate from the transcribed strand than the non-transcribed strand<sup>1,22–25</sup>. The involvement of RAD3 and RAD25 in both transcription and DNA repair suggests a key role for these two proteins in effecting preferential repair of the transcribed strand. □

## Insights into autoregulation from the crystal structure of twitchin kinase

Shu-Hong Hu<sup>\*†</sup>, Michael W. Parker<sup>\*</sup>,  
Jun Yi Lei<sup>‡</sup>, Matthew C. J. Wilce<sup>\*</sup>, Guy M. Benian<sup>‡</sup>  
& Bruce E. Kemp<sup>\*§</sup>

<sup>\*</sup> St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

<sup>‡</sup> Department of Pathology, Emory University, Atlanta, Georgia 30322, USA

MANY protein kinases are self-regulated by an intrasteric mechanism where part of the enzyme's structure directly inhibits the active site<sup>1,2</sup>. This inhibitory structure is called a pseudosubstrate and specific regulators are required to remove it from the active site to allow substrates access. Removal of the pseudosubstrate sequence from members of the myosin light-chain kinase subfamily<sup>1</sup>, including twitchin kinase, activates them but it is not known whether the pseudosubstrate sequence binds to the active site. Native twitchin is a 753K protein (6,839 residues) located in muscle A-bands of the nematode *Caenorhabditis elegans*<sup>3,4</sup> and because of its size has not been easy to study. We have determined the crystal structure, refined to 2.8 Å resolution, of a recombinant fragment (residues 5,890 to 6,262) of twitchin kinase<sup>3,4</sup> that contains the catalytic core and a 60 residue carboxy-terminal tail. The C-terminal tail extends through the active site, wedged between the small and large lobes of the structure and making extensive contacts with the catalytic core which accounts for autoinhibition and provides direct support for the intrasteric mechanism of protein kinase regulation.

Mutants of the *unc-22* gene encoding twitchin have impaired movement, including a 'twitch' of the body surface and disorganized body wall muscle sarcomeres<sup>5</sup>. The structure of the twitchin kinase fragment (residues 5,890–6,262, *M<sub>r</sub>* 40K, 52% sequence identity with chicken gizzard myosin light-chain kinase (MLCK); without insertions or deletions) was determined by the multiple isomorphous replacement method. Crystallization, structure solution and refinement are summarized in Table 1. The backbone structure of the nucleotide-free twitchin kinase catalytic core contains the canonical bilobate protein kinase structure<sup>6–9</sup> (Fig. 1a, b shown in green). Overall, the structure of the catalytic core of twitchin is remarkably similar to the cyclic AMP-dependent protein kinase (cAPK) structure<sup>6,19</sup> (Fig. 1a, b). The C $\alpha$  atoms of the twitchin catalytic core (residues 5,941–6,196) (Fig. 1a, b shown in green) are within 1.9 Å of the corresponding cAPK residues<sup>6</sup> following superpositioning.

The N-terminal sequence to the catalytic core (residues 5,894–5,940) consists of a pair of antiparallel  $\beta$ -strands (S1–S2) and a pair of short  $\alpha$ -helices (H1–H2). The C-terminal autoregulatory sequence beyond the larger lobe (residues 6,197–6,260) extends up through the active site cleft between the two lobes (Fig. 1a, shown in red). It contains three turns of  $\alpha$ -helix (H11), a short single-turn  $\alpha$ -helix (H12) followed by a short  $\beta$ -strand (S13) running antiparallel to  $\beta$ -strand S12.

There are two other reports of protein kinase crystal structures. The main structural differences between the catalytic core of twitchin kinase and cAPK<sup>6,7,10</sup> and the cyclin-dependent kinase 2 (*cdk2*)<sup>9</sup> are as follows. These involve rotation of the two lobes as well as differences in helix positions within the small and large lobes that apparently occur to accommodate the path of the autoinhibitory C-terminal tail between the two lobes. The

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<sup>†</sup> Present address: Centre for Drug Design and Development, The University of Queensland, Brisbane QLD 4072, Australia.

<sup>§</sup> To whom correspondence should be addressed.

small lobe is rotated about 30°, relative to the large lobe, opening the cleft between them without any major secondary structures changes. Inspection reveals this rotation can be achieved by a hinge motion at the adjacent glycine residues (Gly 6,021 and Gly 6,022) allowing part of the C-terminal structure to fit between the two lobes. A model involving rotation about a corresponding pair of glycine residues 125 and 126 in the cAPK structure has recently been proposed to account for the more compact solution structure resulting from the inhibitor peptide PKI(5–22) binding<sup>11</sup>. At least one glycine is conserved in 90% of serine/threonine kinases and 97% of tyrosine kinases<sup>11</sup> and the twitchin structure provides direct evidence for a hinge at this position. The juxtaposition of the two lobes in the twitchin structure more closely resembles the proposed open model of the solution structure of the cAPK without the inhibitor peptide bound<sup>11</sup>. Hinge movements permit protein domains to shift as semi-rigid bodies but are not unique to protein kinases<sup>12,13</sup>.

Some features of the twitchin structure are more similar to cAPK containing bound nucleotide than to the uncomplexed

structure, suggesting that the C-terminal peptide elicits the nucleotide-bound conformation. This is especially noticeable for the canonical ATP-binding loop, G5,949TGAFG5,954 (yellow, Fig. 1a, between S3 and S4). It is shifted 2.5 Å with respect to the position of the flexible loop present in the nucleotide-free cAPK structure<sup>6,10</sup> and its conformation changes to a  $\beta$ -strand, turn,  $\beta$ -strand structure in twitchin similar to the structure of the porcine heart cAPK complexed with MnAMP-PNP and the inhibitor peptide PKI(5–24)<sup>8</sup>. The other large difference in the small lobe concerns H3 but this is probably intrinsic to the amino-acid sequence: its conformation is more similar to the cdk2 structure as H3 in twitchin is longer than helix C (cAPK), helix B (cAPK) does not exist and there is a three-residue deletion (Fig. 1). In addition, helix H3 (twitchin) is rotated 15° about its C-terminus with respect to the underlying strand compared to the equivalent helix C (cAPK). This may be due to differences in the sequences of the side chains that pack into the strand or may be caused by the hinged motion of the domains.

The C-terminal sequence from Leu 6,197 to Asp 6,260 extends

TABLE 1 Statistics for crystallographic structure determination

| Diffraction data                               |  | No. crystals used | Resolution (Å) | No. of measurements | Unique reflections | Completeness (%) | $R_{\text{sym}}^*$ (%) | MFID† (%) |
|------------------------------------------------|--|-------------------|----------------|---------------------|--------------------|------------------|------------------------|-----------|
| Data set                                       |  |                   |                |                     |                    |                  |                        |           |
| Native                                         |  | 5                 | 2.8            | 109,839             | 34,567             | 91.2             | 9.9                    | —         |
| Hg(OOCCH <sub>3</sub> ) <sub>2</sub>           |  | 1                 | 3.1            | 52,668              | 21,122             | 75.6             | 17.5                   | 22.1      |
| PHMB                                           |  | 2                 | 3.7            | 42,116              | 13,036             | 78.9             | 15.8                   | 21.2      |
| KAu(CN) <sub>2</sub>                           |  | 1                 | 3.7            | 47,987              | 14,590             | 88.6             | 7.2                    | 11.1      |
| MIR phasing                                    |  |                   |                |                     |                    |                  |                        |           |
| Derivatives                                    |  | No. of sites      | $R_c$ (%)‡     | Phasing power§      |                    |                  |                        |           |
| Hg(OOCCH <sub>3</sub> ) <sub>2</sub>           |  | 7                 | 52.5           | 1.89                |                    |                  |                        |           |
| PHMB                                           |  | 6                 | 51.3           | 1.61                |                    |                  |                        |           |
| KAu(CN) <sub>2</sub>                           |  | 4                 | 67.9           | 1.01                |                    |                  |                        |           |
| Structure refinement                           |  |                   |                |                     |                    |                  |                        |           |
| $R$ -factor                                    |  | 20.1%             |                |                     |                    |                  |                        |           |
| Resolution                                     |  | 6–2.8 Å           |                |                     |                    |                  |                        |           |
| no. of observations                            |  | 30,296            |                |                     |                    |                  |                        |           |
| r.m.s. deviation of bond length from ideality: |  | 0.015 Å           |                |                     |                    |                  |                        |           |
| r.m.s. deviation of bond angles from ideality: |  | 2.0°              |                |                     |                    |                  |                        |           |

The recombinant protein was overexpressed in *Escherichia coli* as a fusion product with glutathione S-transferase (GST), cleaved with thrombin, purified and crystallized as described previously<sup>15</sup>. The crystals belong to the orthorhombic space group  $P2_12_12$ , with cell dimensions  $a=144.1$  Å,  $b=168.3$  Å,  $c=60.6$  Å. There are three molecules in the asymmetric unit. Diffraction data were collected using a MARRESEARCH area detector with CuK $\alpha$  X-rays generated by a Rigaku RU-200 rotating anode X-ray generator. Derivatives were obtained by soaking crystals for 1 day in solutions of either 2 mM Hg(OOCCH<sub>3</sub>)<sub>2</sub>, 2 mM *p*-hydroxymercuri-benzoate (PHMB) or 10 mM KAu(CN)<sub>2</sub>, dissolved in a buffer of 26% polyethylene glycol 4000, 100 mM Tris-HCl pH 7.5, 0.2 mM MgSO<sub>4</sub> and 10% glycerol. The diffraction data were processed and analysed using programs in the XDS<sup>16</sup> and CCP4 suite (Daresbury Laboratory, Warrington, WA4 4AD, 1979). Heavy-atom sites were located by difference Patterson and difference Fourier syntheses. Phases to 3.1 Å were calculated using the multiple isomorphous replacement (MIR) method, and improved by solvent flattening (CCP4 suite, Daresbury Laboratory) and non-crystallographic density averaging using the DEMON program suite<sup>17</sup>. An initial model comprising 335 residues out of 373 for each of the three molecules in the asymmetric unit was built into the electron density map using the computer graphics program FRODO<sup>18</sup>. This initial model was refined against native data to 2.8 Å by the method of simulated annealing using XPLOR<sup>19</sup>. Electron density maps were then calculated based on the refined model with coefficients  $(2|F_o| - |F_c|)$  or  $(|F_o| - |F_c|)$  and the model rebuilt allowing another 21 N-terminal and 11 C-terminal residues to be included. Following a round of simulated annealing refinement, the crystallographic  $R$ -factor was 25.2% for all data between 8.0–2.8 Å. Finally, several rounds of manual rebuilding and conventional stereochemical least-squares refinement with PROLSQ<sup>20</sup> resulted in the current model. Tightly restrained individual temperature factors (r.m.s. deviation in  $B$ -factor between bonded main-chain atoms of 1.0 Å<sup>2</sup>) were used in the latter stages of refinement. Non-crystallographic symmetry restraints were not used. The current model contains residues 5,894 to 6,260 with no solvent molecules included in the refinement at this stage. The crystallographic  $R$ -factor is 20.1% for all data between 6.0–2.8 Å. The  $\alpha$  positions of the three subunits within the asymmetric unit deviate from the mean by an r.m.s. difference of 0.61 Å for all  $\alpha$  atoms and all three subunits have very similar Ramachandran plots: both indicate that the three molecules are structurally very similar. The number of non-glycine residues that lie outside the allowed regions of the Ramachandran plot are 6, 4 and 4 for subunits 1, 2 and 3, respectively (residues 5,896 and 6,025 in all three subunits; residues 6,094–6,095 in subunits 1 and 2; residues 6,160–6,161 in subunits 1 and 3). All these residues are located in the surface loops. The average temperature factors for subunit 1, 2 and 3 are 13.8 Å<sup>2</sup>, 19.3 Å<sup>2</sup> and 11.9 Å<sup>2</sup>, respectively. The only place where the temperature factors exceed 50 Å<sup>2</sup> is for residues 5,894–5,905 in subunit 2. The twitchin kinase coordinates will be deposited in the Brookhaven Protein Data Bank. Further details of the refined structure will be published elsewhere.

\*  $R_{\text{sym}} = \sum_i |I - \langle I \rangle| / \sum_i I$ , where  $I$  is the intensity measurement of a particular symmetry-related reflection, and  $\langle I \rangle$  is the average of  $I$  over all symmetry-related reflections.

† MFID,  $\sum_i |F_{\text{PH}}| - |F_P| / \sum_i |F_P|$ , the mean fractional isomorphous difference, where  $F_P$  and  $F_{\text{PH}}$  are the protein and derivative structure amplitudes, respectively.

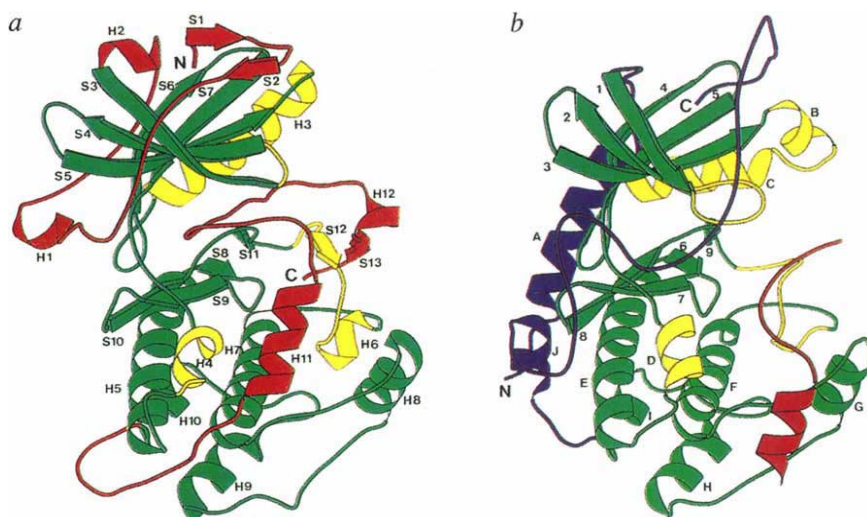
‡  $R_c = \sum_i |F_{\text{PH}}| \pm |F_P| - |F_{\text{H(calc)}}| / \sum_i |F_{\text{PH}}| - |F_P|$ , for all centric reflections.

§ Phasing power, r.m.s.  $\langle F_H \rangle / E$ ;  $F_H$ , heavy-atom structure factor amplitude, and  $E$ , residual lack of closure error.

||  $R$ -factor,  $\sum_i |F_o| - |F_c| / \sum_i |F_o|$ , where  $F_o$  and  $F_c$  are observed and calculated structure factor amplitudes, respectively.

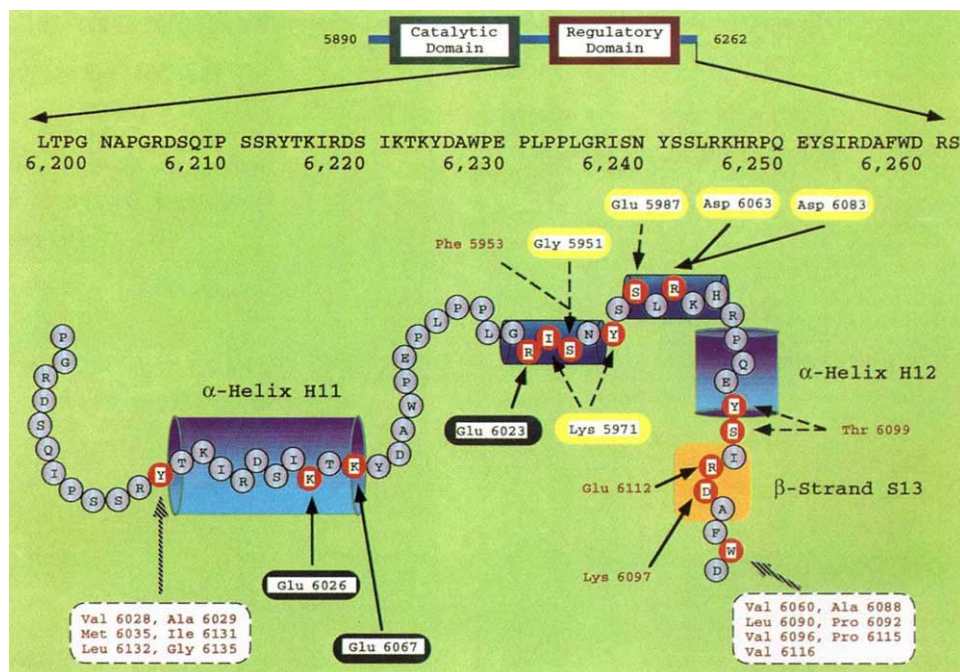


FIG. 1 Ribbon diagrams of twitchin kinase (a) and cAPK (b) (drawn with the program MOLSCRIPT<sup>21</sup>). The coordinates for the cAPK complexed with a peptide inhibitor are from the crystal structure of a binary complex of the mouse recombinant enzyme<sup>10</sup>. Green represents the conserved catalytic core between twitchin kinase (residues 5,941–6,196) and cAPK<sup>10</sup>. The secondary structural elements in twitchin kinase are labelled with  $\alpha$ -helices and  $\beta$ -strands identified as H and S, respectively. The first 4 N-terminal residues and the last 2 C-terminal residues are not apparent in the crystal structure that extends from Arg 5,894 to Asp 6,260. Yellow indicates those regions of the two kinases where the C $\alpha$  atoms differ by more than 2 Å r.m.s. deviation after lobe rotation. The N-terminal (residues 5,894–5,940) and C-terminal (residues 6,197–6,260) sequences of twitchin are shown in red whereas the corresponding sequences in cAPK (residues 10–41 and residues 297–350, respectively) are shown in blue. The inhibitor peptide, PKI(5–24) in cAPK is shown in red. In the large lobe the  $\alpha$ -helix H4 (helix D in cAPK) is shifted 5 Å towards the  $\alpha$ -helix H5 (E helix in cAPK) compared to their corresponding positions in the cAPK structure. The large lobe of twitchin contains an additional  $\beta$ -strand S12 and a short  $\alpha$ -helix H6 instead of a loop in the cAPK or the T loop in the cdk2 structure<sup>9</sup>. There is an interesting double substitution in twitchin with an ion-pair in cAPK replaced by two uncharged residues. In cAPK, Thr 197 is phosphorylated and is bonded to Arg 165<sup>5</sup>; these are replaced by the uncharged Val 6,098 and Leu 6,062, respectively in twitchin, which like the rest of



the MLCK subfamily does not become phosphorylated in the equivalent position. Helices and strands were defined according to the criteria of Kabsch and Sander<sup>22</sup>.  $\alpha$ -helices: H1: 5,921–5,925; H2: 5,937–5,940; H3: 5,978–5,993; H4: 6,023–6,029; H5: 6,037–6,056; H6: 6,108–6,111; H7: 6,118–6,134; H8: 6,144–6,153; H9: 6,168–6,175; H10: 6,188–6,193; H11: 6,215–6,224; H12: 6,249–6,252;  $\beta$ -strands: S1: 5,896–5,899; S2: 5,903–5,906; S3: 5,941–5,951; S4: 5,954–5,961; S5: 5,966–5,974; S6: 6,002–6,007; S7: 6,011–6,016; S8: 6,058–6,061; S9: 6,068–6,072; S10: 6,078–6,082; S11: 6,087–6,090; S12: 6,096–6,099; S13: 6,254–6,257.

FIG. 2 Major autoinhibitory contacts between the C-terminal residues and the catalytic core. The 58 C-terminal residues (6,203–6,260) are shown with single-letter code in circles together with the secondary structural features depicted as follows,  $\alpha$ -helix (large cylinders),  $3_{10}$ -helix (narrow cylinders) and  $\beta$ -strand (yellow square); those residues involved in contacts with the catalytic core shown are in red circles making electrostatic contacts (solid arrows), hydrophobic contacts (hatched arrows) and hydrogen bonds (dashed arrows) to the catalytic core. Every 10 residues are marked in the linear sequence. Within the catalytic core, conserved residues involved in catalysis (yellow boxes), peptide substrate recognition (dark green boxes) and hydrophobic pockets (dashed white boxes) are illustrated. There are several prominent secondary structure features including helix 11 with 3 turns running parallel to helices H4 and H8. Both helix H11 and H4 are shifted away ( $\sim 10.0$  Å) from the position corresponding to the  $\alpha$ -helix in the PKI(5–24) inhibitor peptide (cAPK)<sup>7</sup> (Fig. 1a, b). Within  $\alpha$ -helix H11, Lys 6,224 makes an electrostatic contact with Glu 6,067. This is analogous to the Glu 170 (cAPK) recognition of the P-2 Arg in PKI(5–24). The nearby Lys 6,222 makes a potential contact (3.5 Å) with Glu 6,026 in the H4 helix (D helix (cAPK)) that is conserved in the MLCK subfamily but not in other protein kinases. The side chain of Arg 6,237 forms an electrostatic interaction with Glu 6,023 in the H4 helix. This is analogous to Glu 127 in the cAPK that is responsible for recognition of the P-3 Arg in the inhibitor peptide. However, not every possible substrate contact is exploited. Glu 6,104 (Glu 203 (cAPK) the P-6 recognition site) does not make any electrostatic contacts with basic residues in the C-terminal sequence. The sequence G6,236RISN6,240 forms a short stretch of  $3_{10}$  helix where



the backbone occupies the equivalent position of the ribose 5'-diphosphate of ATP (attempts to soak ATP into twitchin kinase crystals were unsuccessful). A second short stretch of  $3_{10}$  helix with the sequence S6,243 LRKH6,247 is present. The side chain of Arg 6,245 makes electrostatic contacts with Asp 6,063 and Asp 6,083. These conserved residues are equivalent to Asp 166 and Asp 184 in the cAPK and are important for catalysis<sup>8,14,23</sup>. In the cAPK structure, Lys 72 interacts with the conserved Glu 91 (Lys 5,971/Glu 5,987 in twitchin) but in the presence of the C-terminal peptide the side chain of Lys 5,971 forms hydrogen bonds with the backbone carbonyls of Ile 6,238 (2.9 Å) and Tyr 6,241 (2.6 Å). The nearby Ser 6,243 side chain is hydrogen bonded to Glu 5,987 (2.8 Å).

over the surface of the cleft between the two lobes (Fig. 1a, shown in red) and in doing so traverses the entire protein substrate binding groove as well as occupying a substantial region of the ATP-binding site as it wedges between the two lobes. Inspection of the contacts between the C-terminal residues and the catalytic core reveals 47 hydrogen bonds ( $<3.5$  Å) and 351 van der Waals contacts ( $<4$  Å). A number of contacts mimic those expected for peptide substrate side-chain recognition sites, others involve residues in the ATP-binding glycine-rich loop and residues involved in catalysis conserved in all protein kinases<sup>7,14</sup> (Fig. 2).

The twitchin kinase structure provides compelling evidence of the intrasteric model of protein kinase regulation with the autoregulatory sequence exploiting multiple features of the enzyme's active site to inhibit enzyme activity; indeed it mirrors the active site and is more than a simple pseudosubstrate. Preliminary work suggests the mechanism revealed here will be shared by all members of the MLCK and calmodulin-dependent protein kinase II subfamilies and possibly other enzymes. □

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## CORRECTIONS

### The shape, expansion rate and distance of supernova 1993J from VLBI measurements

**N. Bartel, M. F. Bietenholz, M. P. Rupen, J. E. Conway, A. J. Beasley, R. A. Sramek, J. D. Romney, M. A. Titus, D. A. Graham, V. I. Altunin, D. L. Jones, A. Rius, T. Venturi, G. Umana, R. L. Francis, M. L. McCall, M. G. Richer, C. C. Stevenson, K. W. Weiler, S. D. Van Dyk, N. Panagia, W. H. Cannon, J. Popelar & R. J. Davis**

*Nature* **368**, 610–613 (1994)

In this letter some of the references were incorrectly numbered (details available from the first author). □

### Water-based non-stick hydrophobic coatings

**Donald L. Schmidt, Charles E. Coburn, Benjamin M. DeKoven, Gregg E. Potter, Gregory F. Meyers & Daniel A. Fischer**

*Nature* **368**, 39–41 (1994)

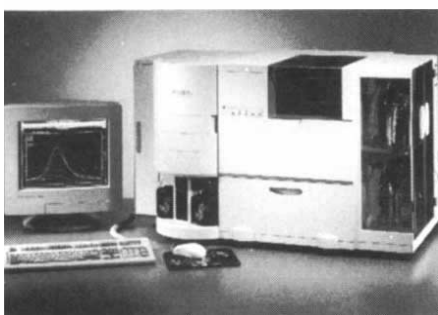
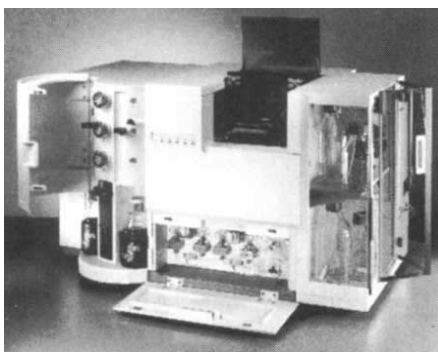
THE chemical nomenclature used for monomer A in this letter should have been correctly given as 2-(perfluorooctyl)ethyl methacrylate (recommended common name) or 1-decanol, 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10 - heptadecafluoro - methacrylate (*Chemical Abstracts* complete name). □



# Molecules in motion

New tools for the chromatographer include an analytical workstation for the characterization and analysis of biomolecules by immunoassay and chromatography, and an assortment of columns, pumps, detectors and accessories.

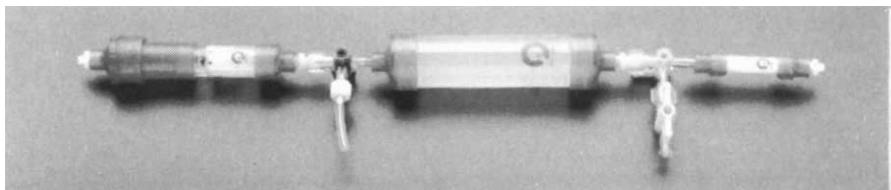
THE new Integral **automated micro-analytical workstation** from PerSeptive Biosystems for the characterization and analysis of biomolecules combines immunoassay and chromatogram technology, hardware and software to optimize the development of analytical methods and the performance of routine assays (*Reader Service No. 100*). The company's Immuno-Detection perfusion immunoassay and perfusion chromatography technologies are central to the performance of the workstation. In addition to supporting routine immunoassays, the workstation introduces automated, two-dimensional assays combining the techniques of immunoassay (IA) and



**Integral microanalytical workstation.**

liquid chromatography (LC). As such, Integral performs automated IA-LC, IA-IA and LC-LC. This benchtop system incorporates an ultraviolet/visible spectrophotometric detector, although it can be configured with a fluorescence or chemiluminescence detector. Other design features include an autosampler with a 105-sample vial capacity, on-board dilution and addition of reagents, a sample cooling feature for the preservation of labile samples, and pumps that can operate at flow rates of  $10 \mu\text{l min}^{-1}$  up to  $10 \text{ ml min}^{-1}$ . Integral's workstation software covers three areas: control of the instrument, methods development and the analysis of data.

NATURE • VOL 369 • 16 JUNE 1994



**Quickpure kit for the purification of polyclonals.**

Quickpure is a kit from Sterogene Bioseparations for the **purification of polyclonal antibodies** that involves immobilization of the peptide/antigen and subsequent purification, buffer exchange and concentration (*Reader Service No. 101*). It can also be used for the purification of monoclonal antibodies, provided that an antigen to the antibody is available. Elution of the antibody from the affinity column utilizes a non-denaturing elution buffer, which operates at neutral pH. The immobilization and purification procedures are said to take less than 3 h. Sufficient reagents, including resins that are supplied as prepacked columns, are provided for performing 25 purifications. A larger system for the purification of 100 mg antibody per cycle is also available.

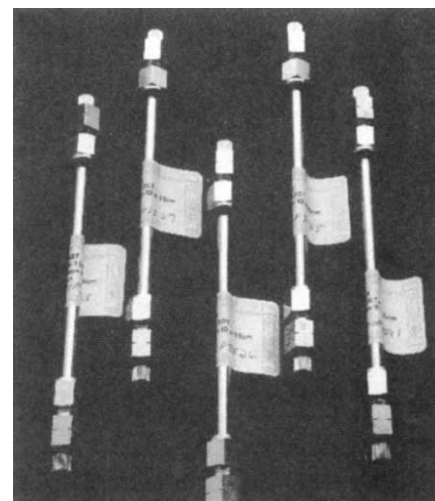
Proteinase K — used especially in the isolation and purification of nucleic acids, for example in plasmid preparations, in the activation of restriction and DNA-modifying enzymes, in the pre- and post-treatment of PCR samples — can now be obtained in an immobilized form from E. Merck (*Reader Service No. 102*). The company says that its **immobilized proteinase K** is reusable, provides stable, covalent bonding via the free-moving tentacles, can be removed by centrifugation/filtration, and is free of DNases, RNases and phosphatases.

Axxiom Chromatography's new Peak-Trak SC **solvent conservation module** connects to any chromatographic detector and is designed for the automatic recycling of clear solvent eluting from the column, in isocratic HPLC (*Reader Service No. 103*). 'On-the-fly' baseline slope detection finds and cuts all peaks, regardless of rising or falling baselines, or peak size range, says Axxiom. It can also place tick marks in chromatograms at each peak cut point, allowing run-by-run visual performance checks.

## ■ Columns

For the **analysis of mono- and oligo-saccharides**, Tosohaas offers its new TSKgel Amide-80 normal-phase column (*Reader Service No. 104*). The column is packed with  $5 \mu\text{m}$  silica bonded with carbamoyl groups.

According to Tosohaas, this packing does not react with reducing sugars, and can be used for separations at higher temperatures. The column is said to provide an alternative to amino-bonded phase columns when using normal-phase liquid chromatography to sepa-



**Columns for carbohydrate separations.**

rate saccharides. In addition, Tosohaas offers a new polymeric TSKgel OD-2PW polymeric, octadecyl reversed-phase column. This column features a  $5 \mu\text{m}$  methacrylate-based polymer packing, and is designed for use from pH 2–12.

Hamilton offers a range of polymeric, **reversed-phase narrowbore HPLC columns** (2.1-mm internal diameter) in 150- and 250-mm lengths (*Reader Service No. 105*). These columns are designed to allow the user to achieve greater sensitivity with less sample, while decreasing solvent consumption and reducing the cost of waste disposal. The following stationary phases are available: PRP-1, PRP-3, PRP-X100, PRP-X200, PRP-X300, PRP-X400 and RCX-10.

Amicon's Pirkle-type  $\pi$ -acceptor **chiral stationary phase** for preparative chiral chromatography is now available in bulk quantities (*Reader Service No. 106*). R-DNPG is immobilized on the company's 100 Å,  $10 \mu\text{m}$  spherical silica, and is intended for

use in dynamic axial compression columns. Amicon says that, under normal-phase conditions, the enantiomers of many compounds, such as benzodiazepines and their derivatives, can be resolved at loadings up to milligrams per millilitre of column volume. This packing is available in analytical columns and bulk quantities in 100-mg, 1-kg and 10-kg containers.

IonClear BigBead from Sterogene Separations is designed to allow the **direct capture of antibodies/proteins** from the fermentation/cell culture harvest without the need for a 3–5-fold dilution (*Reader Service No. 107*). It replaces diafiltration with an ion absorption process. Both cations and anions can be captured from solutions. Its large bead size and special surface modifications are optimized for small-ion binding without binding protein. It also removes Phenol Red from cell culture harvest. IonClear BigBead is said to be stable over the pH range of 1–14 and can be autoclaved.

Bio-Rad's new **prepacked ion-exchange column** line offers researchers a means of performing medium-pressure chromatography separations of biomolecules, including proteins, peptides and polynucleotides (*Reader Service No. 108*). Columns are available in both strong anion exchange and strong cation exchange. Four different sizes — 2, 5, 10 and 20 ml — are available. Bio-Rad says that the lifetime of the columns can be extended by using the Top Off resin replacement kit.

The new EcoCART 125-3 **glass HPLC cartridge column** from EM Separations has been designed to reduce solvent consumption (*Reader Service No. 109*). It consists of a combination of a pressure-resistant glass column with PEEK end fittings housed in a stainless steel cartridge holder with two view ports. A system of manu-CART guard column adapters and cap nuts are intended to ease configuration and assembly. The smooth, transparent glass surface enables the viewing of separations of coloured substances as they occur.

### ■ Pumps

While the LC-10AT **pump** from Shimadzu is being billed on the one hand as a stand-alone starter pump for researchers on a budget, it can, on the other, form part of a



**Pump it up with the LC-10AT.**

fully automated HPLC system for routine analyses (*Reader Service No. 110*). The pump provides both types of gradient elution: a high-pressure mode with three solvents and a low-pressure mode with four solvents.

The new CMA/250 LC pump from CMA Microdialysis is a **metal-free, dual-piston, high-pressure liquid delivery pump** intended for separations of labile molecules in biomedical applications and for ion chromatography (*Reader Service No. 111*). All 'wetted' surfaces in the pump head are nonmetallic and are made of PEEK, PTFE, ruby and sapphire. The low displacement volume (16 µl per stroke) is said to provide uniform flow rates from 10 µl min<sup>-1</sup> to 3 ml min<sup>-1</sup> in 10-µl steps. Pressure stability is automatically maintained by a self-learning system each time the pump is started. CMA says that pulse compensation is effectively made by a two-point calibration between two preset flow rates. Pressure pulsations are normally below 1 per cent, according to CMA, although maximal suppression of pressure pulsations in the lowest flow-rate range can be achieved by the installation of an optional pulse damper within the chassis of the pump.

### ■ Detectors

The ED40 **electrochemical detector** from Dionex, suitable for applications in ion chromatography and HPLC, can be used in the detection of a range of analytes including carbohydrates, catecholamines, anions and cations (*Reader Service No. 112*). Three

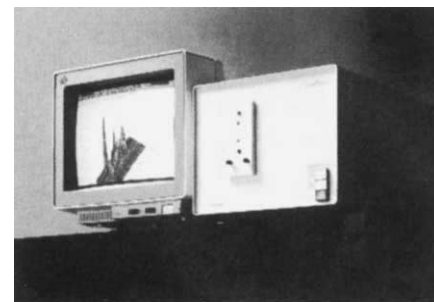


**Dionex's ED40 electrochemical detector.**

modes of detection are provided within a single electrochemical instrument, including integrated amperometry, d.c. amperometry and conductivity, plus an added operational mode, cyclic voltammetry. Stated detection levels are in the low-picomole range for carbohydrates and the low-femtomole range for catecholamines. The small-diameter working electrode and the thin-channel design of the cell, are intended to provide a high signal-to-noise ratio during analyses. During amperometric operation, the ED40 can be used to monitor pH, and during conductivity operation cell temperature can be monitored.

Gilson's new 160 **diode-array detector** offers an ultraviolet/visible detection range

from 190–700 nm, a large number of array elements and an optical design based solely on reflective optics (*Reader Service No. 113*). The detector features a 512-element detector that is designed to allow compounds with fine spectral features, such as aromatics, to be depicted accurately. Control and data acquisition are provided by the Microsoft Windows-based Gilson 160 controller software or through the company's HPLC



**Gilson's diode-array detector.**

system controller software. Scan data are collected and stored each time the array is read. Software is available that allows the user to view chromatograms, spectra, or both, simultaneously. Multidimensional plots with rotation and scaling can also be viewed. Plots can be displayed in a variety of formats, including three-dimensional (3-D), and data from different runs can be viewed and compared onscreen. Chromatograms, spectra, and 3-D plots can be imported into other Windows applications to create customized reports, presentations and publications. Three different flow cells are available, including biocompatible.

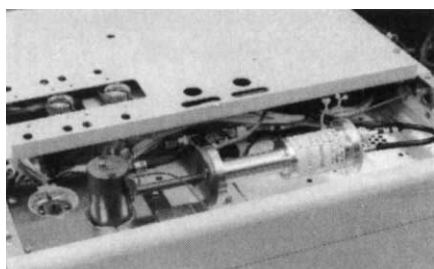
The Decade **digital electrochemical amperometric detector**, developed by Antec and available from Presearch, offers three modes of operation for use in liquid chromatography (*Reader Service No. 114*). In addition to the conventional d.c. mode for amperometric detection, the instrument offers a pulse mode for the determination of carbohydrates and sugars in alkaline conditions and a scan mode for the determination of electrochemical activity in new or uncharacterized samples. Decade is available with a variety of electrodes, including glassy carbon, gold and platinum, and incorporates a temperature-controlled cabinet for the injector, column and cell. The instrument is equipped with several I/O connections to allow linking with most instruments: it can be controlled by a computer ('slave mode') via the optional RS-232 connector, or it itself can control several other instruments ('master mode').

The 474 is the new **scanning fluorescence detector** from Waters (*Reader Service No. 115*). A dual monochromator design enables the excitation and emission wavelengths to be programmed independently, providing users with the ability to optimize detection parameters for com-



pounds of interest. According to Waters, the Raman signal-to-noise ratio is greater than 200 for this model. Programmable features that are built in include automatic switching of wavelengths during a run, scanning of excitation and emission wavelengths, storage of up to 10 methods with 64 steps in each method, a lamp 'standby' function, and three selectable bandwidths.

Varian's new **pulsed-flame photometric detector**, intended for the detection of sulphur and phosphorus compounds, is said to be compatible with all types of injectors and columns (*Reader Service No. 116*). The design of the detector, which is based on the research of Aviv Amirav at the University of Tel Aviv (*Analyt. Chem.* **65**, 539-555; 1993), does not sustain a continuous flame. Rather, the ignited flame 'propagates' through the detector, combusts the sample molecules, and then 'self-terminates' after the combus-



**Pulsed-flame photometer for GC.**

tible mixture is burned, creating a 'pulse'. With this detector, Varian claims that hydrogen/air gas consumption is lower than with standard flame photometric and chemiluminescence detectors, and peak tailing for phosphorus compounds is eliminated.

## ■ Injectors/collectors

The new Model 234 **autoinjector** from Gilson performs routine injection of ready-prepared samples into an analytical HPLC column (*Reader Service No. 117*). The instrument also dilutes, add internal standard and re-injects standards. Gilson says that the instrument has been designed to provide easy access to the sample rack, injection



**Autoinjector for routine HPLC.**

valve and the hydraulic connections. The instrument is available with an optional Peltier unit for controlled heating and cooling of samples.

The new Model 2128 **fraction collector** from Bio-Rad is designed for use in both analytical and preparative applications in combination with a low-pressure chromatography or HPLC system (*Reader Service No. 118*). A range of racks and adaptors is available to accommodate most sizes of collection vessel — from microtitre plates and standard tubes to large bottles. The software enables programmed fractionation by drop-counting, time, time-windows (maximum 20) and/or peak detection. The Model 2128 is said to be able to fractionate at flow rates up to 100 ml min<sup>-1</sup>, and to collect double peaks separately. The device can be used in cold rooms. An optional diverter valve performs both diversion of unwanted eluent into waste and diversion of flow between fraction changes. The device works with the Econo system through a dedicated Econo software option. It also operates in a standard mode for use with other low-, medium- or high-pressure chromatography systems.

Gilson's FC 205 **fraction collector** is designed for applications requiring routine collection in time and drop modes (*Reader Service No. 119*). With the FC 205, which is priced at US\$995, up to 10 collection win-



**FC 205 offers time/drop collection and a range of rack options.**

dows can be added in either mode to divert non-desired eluent to waste, in this way, making optimal use of the collection tubes. The collector can be used as a stand-alone instrument or may be integrated with a Gilson HPLC or LC system, or that of another manufacturer. Key features of the FC 205 include, a stationary rack system to eliminate jams, drips and spills; x-y stepper motor technology; metal internal drive components for reliable operation, even in cold rooms; and a keyboard and case that are resistant to chemicals. Collection parameters may be edited during a run, and the most recent set of parameters is automatically stored in the memory. A range of rack and tube options is provided, with 23 different racks (three that are thermostatic), 10-, 12- and 13-mm test

tubes, standard and deep-well microtitre plates, microcentrifuge tubes, scintillation vials and collection via Teflon funnels. An optional multi-column adapter enables simultaneous collection from up to eight separate columns.

## ■ GPC/GC

The PL-GPC210 from Polymer Laboratories is designed for the routine high-temperature GPC **analysis of polyolefins and other crystalline polymers** (*Reader Service No. 120*). As polymers of this type require operational temperatures approach-

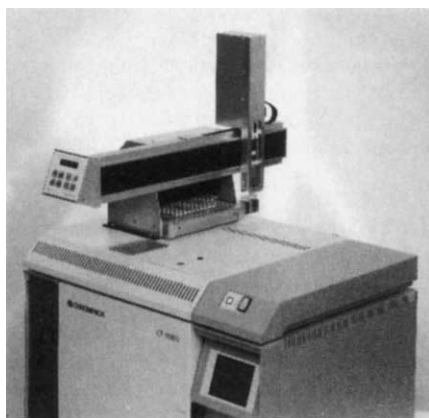


**High-temperature GPC system.**

ing their melting points to maintain solubility of the sample, the instrument has been designed to operate at temperatures up to 250 °C. Design features of this instrument include a temperature-controlled solvent delivery system and a programmable, dual-zone, 40-vial autosampler that maintains the pre-filtered in a lower-temperature 'standby' zone before it enters the stirred, hot, pre-injection zone. This feature is designed to minimize the possibility that the sample will degrade prior to analysis. An RI detector completes the system.

Shimadzu's benchtop **gas chromatograph/mass spectrometer**, the QP 5000, which was launched last year, is now available as an electron ionization/chemical ionization (EI/CI) version, including an exchange ion source for chemical ionization (*Reader Service No. 121*). This latest version features a larger capacity turbomolecular pump as standard, and a second rotary pump dedicated to the reactant gas system, as well as devices to measure pre- and high vacuum, that is, a Pirani gauge and ionization gauge. Using methane, isobutane and ammonia, the most common reagent gases can be fed to the ion source via CI gas controller. The stated sensitivity is described by a signal-to-noise ratio of 30:1 for a quasimolecular peak of 100 pg benzo-phenone when scanning from m/z 100 to 250 using a scan interval of 0.6 s.

Completely controlled by the CP-9001 gas chromatograph, the new CP-9010 **automatic liquid sampler** from Chrompack enables round-the-clock batch processing of up to 105 samples for analyses by gas



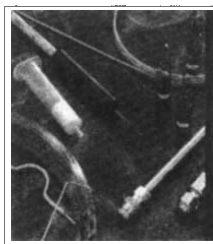
**Round-the-clock automatic sampling for GC analysis.**

chromatography (*Reader Service No. 122*). Cooling or heating of the sample tray preserves biological samples or prevents samples from crystallizing. Injection in steps of 0.1 µl up to 10 µl into packed, splitter, splitless, wide-bore or on-column is possible. The sampler also allows composite injection for routine internal standard calibration, or any specific injection technique, such as 'sandwich', 'air plug' or 'hot needle', into two injection ports. The device is electrically, rather than pneumatically, controlled.

#### ■ Odds and ends

An HPLC solvent filter-degasser system, designed to enhance the performance of pumps and detectors by removing suspended particles and degassing solvents, can now be obtained from Lazar Research Laboratories (*Reader Service No. 123*). The FG-256 system screws directly onto a standard solvent bottle and is designed to filter and degas as much as one gallon of solvent without the need for refilling or operator intervention. All components of the device are made out of Teflon, making it suitable for use with both aqueous and organic solvents. The unit uses standard 47 mm filter membranes and connects directly to a vacuum source.

Supelco has released its first **catalogue of separations products** since becoming part of the Sigma-Aldrich-Fluka Group (*Reader Service No. 124*). More than 9,000 products for gas chromatography, high-performance liquid chromatography (HPLC), low-pressure liquid chromatography (LC), thin-layer chromatography and capillary electrophoresis are featured.



**On sale at Supelco.**

Supelco's new line-up includes new sample handling technologies, high-purity solvents, a selection of affinity, ion-exchange, adsorption/partition and size exclusion media for low-pressure LC, and HPLC

and low-pressure LC columns. Also included are new chemical standards for environmental, petroleum/chemical and other analyses, buffers and reagents, sample preparation equipment, and products for general laboratory use. More than 500 application chromatograms with analysis conditions are also featured.

The analytical instrument supplier, Varian, is now offering its **chromatography parts and supplies catalogue on diskette** (*Reader Service No. 125*). AutoCat, which is available free of charge, is designed to be more flexible than a paper catalogue, enabling users quickly to scroll through part numbers and view descriptions of the products and their prices. As items and quantities are selected, an order form is automatically filled in, which can later be edited, printed or faxed directly from a personal computer. AutoCat will run on any computer running Windows 3.1 software with at least 4 MB main memory, 5 MB hard disk storage space and a 3.5-inch disk drive.

Chrompack has introduced a new brochure covering its line of **capillary columns for gas chromatography** (*Reader Service No. 126*).



**Chrompack's capillary columns for GC.**

In all, over 300 different columns are featured, including Chrompack's latest Ultimet Plot columns —made of an inert material. Six chapters cover everything from tailor-made columns and fused silica columns to column accessories.

Shandon's new **catalogue of materials and columns for liquid chromatography** can now be obtained from Life Sciences International (*Reader Service No. 127*). Included among these are Hypersil, Hypersil ODS and other bonded phases, the company's new generation of Hypersil BDS phase, columns for environmental research, materials and columns for preparative chromatography, and columns and cartridges for sample preparation. Additionally, the catalogue lists other speciality columns, including those for affinity chromatography and biochromatography for use in the separation of peptides, proteins and nucleotides, and Hypercarb carbon graphite columns designed for the separation of closely related and highly polar compounds. □

*These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.*

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# A moral question for patent legislation

Richard Price and Simon Cohen

**In considering the grounds on which to award patents for applications of germline gene therapy, the European Patent Office may have to decide on the basis of "morality". This should not be its job.**

THE European Patent Office (EPO) is facing a delicate decision: whether to allow an application to patent germline gene therapy. A research group at the University of Pennsylvania (see *Nature* 368, 572; 1994) has outlined a technique of altering the germ line of animals by, among other things, arranging that the testes should produce only genetically engineered spermatozoa, so that the offspring inherit only the specified genes. The detailed scope of the patent application will become known only when it is published. But the principle is clear. Unlike somatic gene therapy, in which the alterations affect only the patient (in cystic fibrosis sufferers, for example, only the cells lining the patient's lungs), germline gene therapy affects the patient's offspring. This raises concern about the possible abuse of such treatment.

The European Patent Convention is an agreement signed by all EC states, Austria, Sweden and Switzerland. National patent laws in all these countries have been amended to conform with it. Under its provisions, before granting a patent, the EPO has to consider requirements of novelty, inventive step, sufficiency of disclosure, industrial application and, most controversially, morality.

**Novelty.** An invention must be new in the sense that it must not form "part of the state of the art". The test is one of worldwide novelty, so that a prior disclosure anywhere will prevent the grant of a patent. Under US law, however, applicants have one year after disclosure in which to file a patent application. In contrast to somatic gene therapy (more than 50 trials of which are planned or under way), germline gene therapy is likely to be a novel process according to this definition.

**Inventive step.** An invention has an inventive step if "having regard to the state of the art, it is not obvious to a person skilled in the art". The EPO must be satisfied that, given the state of the art at the time of the application, the invention does not involve the exercise of any ability beyond that to be expected of the ordinary person "skilled in the art".

**Disclosure of the invention (sufficiency).** The application must also disclose the invention in a manner sufficiently clear

and complete for it to be carried out by a person skilled in the art. This is to ensure that the application contains sufficient technical information to enable that person to put the invention into practice, and to enable the reader to understand the contribution that the inventor has made.

**Industrial application.** An invention must also be capable of industrial application: capable of being "made or used in any kind of industry, including agriculture". However, methods for treatment by surgery or therapy are not considered to be capable of industrial application. Therapy implies the curing of a disease or malfunction of the body and covers prophylactic treatment, for example immunization. Treatments which are not curative are therefore patentable. A borderline case was the patent for killing body lice *in situ*, which was allowed by the English High Court on appeal from the UK Patent Office. This exception covering methods for treatment will be of little help to the EPO in deciding on patentability for germline gene therapy. Whereas a patent for somatic gene therapy itself may come within the exception (for example the cystic fibrosis therapy referred to above), germline gene therapy, not necessarily having a curative effect and affecting later generations, would probably not. So the EPO will have to consider the morality question.

**Morality.** The European Patent Convention precludes the patenting of inventions, the publication or exploitation of which would be "contrary to *ordre public* or morality". The EPO guidelines state that this provision is to exclude inventions likely "to induce riot or public disorder, or to lead to criminal or other generally offensive behaviour". The example given, unhelpfully, is a letter-bomb!

On the limited information available at the moment, we understand that the "germline" patent is very broad in its range of applications. It relates to animals, human beings included. It aims to cover the curing of a particular gene defect as well as the removal of sperm (where there may be no particular gene defect) and replacement with "superior" sperm. Some of these applications will inevitably seem more "immoral" than others.

According to the doctrine of the separation of powers, a moral decision is not a decision that patent offices should be called on to make. It should be reserved for the political power. When the decision

has been made, it should be formulated in primary legislation, with the rules and guidance needed for its application in individual cases clearly set out. While application of the law may involve a moral judgment in a marginal case, patent offices should not have to make a moral choice. They apply a series of essentially technical tests to decide whether a patent should be granted. Requiring them to decide this moral question is beyond their competence and may result in them failing to give a proper moral judgment, and in muddling the technical assessment.

The EPO had to consider the issue in the application for the Oncomouse, a mouse developed at Harvard University whose offspring develop tumours soon after birth. Balancing the suffering of animals and possible risks to the environment against the invention's usefulness, the EPO decided that the "Oncomouse's purpose of facilitating cancer research and treatment was of paramount importance for the welfare of mankind" and granted the application. But the patent is now being opposed.

More guidance is clearly required for patent offices and inventors. One possible way forward is to set up an ethics committee. If a patent raises morality concerns when it is examined then it could be granted subject to conditions as to its exploitation. The patent owner would then have to clear its mode of exploitation with the ethics committee in advance. There are two main reasons why this suggestion makes sense: first, because the vast majority of granted patents are never commercialized — why should the EPO spend considerable time dealing with such issues when the patent may never be exploited? Second, in many instances (as in the germline application) the patent is broad. By involving the ethics committee at a later date, when commercialization is in sight, the EPO would be able to see how an invention would practically be exploited before making its final decision.

Whether or not this particular idea finds favour, some changes will surely have to be made. Otherwise, the EPO is bound to face many more biotechnology applications giving rise to unnecessary controversy, and causing the public to ask whether patent law is coping with the pace of technological change. □

The authors are solicitors at Taylor Joynson Garrett, London EC4Y 0DX, UK.

This is the first of an occasional series of articles on intellectual property rights and the law.

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## Categories of paper

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